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Contents – Sommaire – Inhaltsverzeichnis

Vol. 35

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Die Verdünnungsanämie

Tierexperimentelle Untersuchungen über den Einfluß des Blutvolumens auf die Erythropoese

Hj. Becker und D. Spengler

Untersuchungen des Blutvolumens bei Patienten mit chronischen Anämien unter Transfusionsbehandlung (5) und bei Polycythämiekranken nach Aderlässen und Behandlung mit Radiophosphor (4) hatten uns zu folgenden Vorstellungen über die Blutregulation geführt: Zellkonzentration und Blutvolumen (BV) werden weitgehend unabhängig voneinander reguliert, stehen aber in enger Wechselbeziehung zueinander. Die Größe des BV ist eng verknüpft mit der anatomisch und funktionell bestimmten Kapazität des Gefäßsystems und paßt sich ihr durch Änderungen des Plasmavolumens (PV) an. Eine Größenänderung des PV wirkt sich aber – im Sinne der Bluteindickung oder der Verdünnung – auf die Zellkonzentration aus. Die Regelung des Gesamtvolumens, das aus Kreislaufgründen möglichst konstant gehalten wird, ist dabei der Regelung der Erythrozytenkonzentration übergeordnet. Für die Sauerstoffversorgung des Gewebes sind aber in erster Linie die Erythrozyten- bzw. Hämoglobinkonzentration im Blut von entscheidender Bedeutung, nicht die Größe des BV bzw. die Gesamtmenge zirkulierender Erythrozyten (Erythrozytenvolumen = EV). Die Sauerstoffkonzentration im Gewebe bestimmt aber das Ausmaß der Erythropoese im Knochenmark unter Einschaltung noch unbekannter Rezeptoren, erythropoetinbildender Gewebe und humoraler Faktoren.

Somit ist zu erwarten, daß eine Abnahme der Zellkonzentration im Blut (Ery/mm³) auch dann zu einer Aktivierung der Ery-

Durchgeführt mit Unterstützung der Deutschen Forschungsgemeinschaft, Bad Godesberg.

thropoese führt wenn das EV konstant bleibt Solche Bedingungen lassen sich experimentell durch Infusionen großer Mengen eines Plasmaexpanders erzeugen. Es resultiert dann eine Verdünnungsanämie mit unverändertem EV

Erslev (18) fand nun aber bei derartigen Versuchen bei Kaninchen weder einen Anstieg der Reticulozyten noch eine Aktivierung der Erythropoese im Knochenmark. Er vermutete, daß die Abnahme der Zellkonzentration durch eine erhöhte Zirkulationsgeschwindigkeit soweit ausgeglichen werden könne, daß im Gewebe keine Hypoxie entstünde. Dieser an 4 Kaninchen erhobene Befund gilt seitdem als grundlegend für die Theorie der Erythrozyten regulation (12 29) Da es uns unwahrscheinlich erschien, daß eine erhebliche Senkung der *Hämoglobin und Erythrozytenkonzentration* durch Änderungen der Kreislaufgrößen völlig kompensiert werden könne haben wir die Versuche von Erslev nachgearbeitet. Dabei ergab sich, daß die Verdünnung des *Blutes* allein zu einer starken Aktivierung der Erythropoese mit vorübergehender reaktiver Polyglobulie führt.

Methoden

Versuchstiere 10 ausgewachsene männliche Kaninchen, 4,5 bis 5 kg schwer Trockenkost (Kombinefutter Rabbit Rations, Hope Farms) Wasser ad lib. – 3 Tiere starben im Stadium der maximalen Hypervolämie. Die Untersuchungen bei 12 Tieren wurden ausgewertet Vorbehandlung K 1 2 4: bis Versuchsbeginn 6 × Vit. B 12 30 γ und 50 mg Fe (Ferri-Sorbitol-Zitrat Komplex, Jectofer®) i. m. – K 3, 5, 6 je 60 γ Vit. B 12 und 100 mg Fe. K 8 15 je 30 γ Vit. B 12 und 50 mg Fe vor Versuchsbeginn und 6 × innerhalb der ersten 4 Versuchswochen. – Die Versuche wurden erst begonnen, nachdem konstant Hb werte in der Vorbeobachtungsperiode festgestellt waren.

Dextran Macrodex® 6%, mit physiologisch ausgewogener Elektrolytlösung[1]) Infusionen in die marginale Ohrvene 2 täglich, meist je 18 ml/kg. Diese Dosis wurde von den meisten Tieren gut toleriert; größere Infusionsmengen (25 ml/kg) führten nach einigen Tagen zum Tod der Tiere

Blutuntersuchungen. Hämoglobin (Hb) *Cyanhämoglobinmethode (Doppelbest.)* Erythrozyten Zählung in *Thoma-Zählkammer (Doppelbest.)* Reticulozyten (Retic.) Färbung mit 1% Brillantkresylblau in 0,9 NaCl-Lösung Mischung in der *Pipett* Färbung in feuchter Kammer 20 min, Ausstriche Auszählung von je 2000 Erythrozyten Angabe der Retic in ‰ der Erythrozyten. – Hämatokrit (Hk): nach einem Vorschlag von Schmidt (48) wurden Hk Röhrchen verwendet die außer der Hk-Bestimmung auch die Aktivitätsmessung chrommarkierter Erythrozyten in Vollblut und *Erythrozytensediment gestatten, da sie ein konstantes* Volumen (0,5 ml) besitzen[2]) Höhe 65 mm Teilungslänge 50 mm. Zentrifugieren 60 min, 3000 UpM Radius der Zentrifuge 18 cm. Korrektur für trapped plasma nach den für diese Röhrchen ermittelten Werten (48) Alle Hk Werte sind nach dieser Korrektur angegeben – Plasmaeiweiß

[1]) Wir danken der Firma Knoll AG Ludwigshafen für die Überlassung der Versuchsmengen

[2]) Hersteller Rhema-Glas, Hofheim/Taunus.

Biuret-Methode nach Weichselbaum. Im Gegensatz zu den Untersuchungen anderer *Autoren mit einem amerikanischen Dextranpräparat* (28) *wird diese Reaktion nicht* durch eine Präzipitation von Macrodex beeinträchtigt (56) – Serumeisen: Bathophenanthrolinmethode (Ferropack®) – Knochenmarkuntersuchung: Punktion der oberen medialen Tibiafläche (62). Ausstriche, Färbung nach Pappenheim. Auszählung von je 1000 Zellen der myeloischen Reihe und der darauf entfallenden Zellen der Erythropoese (M/E-Index).

Blutvolumenbestimmung: Entnahme von 7 ml Blut mit geringem Heparinzusatz. Bei wiederholten Bestimmungen Messung der Restaktivität. Zentrifugieren 5 min mit geringer Tourenzahl (wegen der vermehrten Agglutinationsneigung der Retic. (34)). Erythrozytensediment mit 7 µC $Na_2Cr^{51}O_4$ in 0,9% NaCl-Lösung 30 min bei 37° inkubiert unter gelegentlichem Umkippen. Zugabe von 25 mg Ascorbinsäure (43) und eisgekühlter 0,9%-NaCl-Lösung. Einmaliges Waschen mit geringer Tourenzahl. Auffüllen bis zum Ausgangsvolumen. Nach Abtrennung des Meßstandards (Doppelbest.) Injektion einer exakt definierten Menge der Erythrozytenaufschwemmung. Blutentnahme nach 1 Std. (Doppelbest.)

Alle Aktivitätsmessungen wurden in den beschriebenen Hk-Röhrchen durchgeführt (Bohrloch-Szintillationszähler Frieseke & Hoepfner 488 mit 10 cm starker Bleiabschirmung)[1]; die Erythrozytensäule bleibt dabei immer im optimalen Meßbereich des Bohrlochkristalls. Aktivitätsmessung des 1-Std.-Wertes und des Meßstandards (nach exakter *Entfernung des Überstandes*) und von *0,2 ml des Standard-Überstandes* (zur Korrektur für die Aktivität des trapped plasma). Berechnung des EV nach Gray und Sterling (nach Korrektur für trapped plasma), wobei auch der durch die laufenden Hk-Bestimmungen entstandene Blutverlust (je 0,5 ml) berücksichtigt wurde (Einzelheiten bei 56). Berechnung des BV aus EV und (korrig.) Hk. Wie in-vitro-Versuche zeigten, wird der Hk auch durch hohe Macrodexkonzentrationen nicht beeinflußt (56).

Bestimmung der Lebenszeit chrommarkierter Erythrozyten: Der 1-Std.-Wert der BV-Bestimmung wurde als 100%-Wert für die Lebenszeitbestimmung eingesetzt. Hk-Bestimmungen in regelmäßigen Intervallen, Messung der Aktivität im Erythrozytensediment der Hk-Röhrchen, Umrechnung auf 1 ml gepackte Zellen. Messungen im Vollblut waren wegen der im Versuchsverlauf auftretenden Plasmavolumenänderungen nicht möglich.

Anreicherung jugendlicher Erythrozyten: Die Untersuchungen wurden 5 Tage nach Beginn der Reticulozytose, 7 Tage nach einer vorangegangenen Chrommarkierung durchgeführt. Der relative Anteil jugendlicher Erythrozyten war infolge der seit mehreren Tagen erhöhten Reticulozytenzahl bereits stark vermehrt, erkennbar an dem deutlichen Abfall der Aktivität der zuvor markierten Erythrozyten (vgl. Abb. 8). Wegen ihrer geringeren Dichte können Reticulozyten und jüngere Erythrozyten durch Zentrifugieren in der oberen Schicht weiter angereichert werden (42), allerdings bei Kaninchen nicht so gut wie in menschlichem Blut (63). *Aus der Differenz der Aktivität des Gesamtblutes* und der oberen Schicht nach Zentrifugieren (jeweils umgerechnet auf 1 ml gepackte Zellen) ergibt sich der Anteil älterer Zellen (die 7 Tage zuvor markiert wurden). Die Aktivität der oberen Schicht lag um 30% niedriger als die des Gesamtblutes. Auf eine bessere Abtrennung und damit eine noch größere Ausbeute jüngerer Zellen durch längeres und schärferes Zentrifugieren wurde wegen der bekannten Agglutinationsneigung der Retic. (34) verzichtet. Das Blut der oberen Schicht, das so mit jüngeren Erythrozyten angereichert war, wurde neu zur Bestimmung der Lebenszeit mit Cr^{51} markiert und nach vorheriger Kreuzprobe auf andere Kaninchen übertragen.

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[1]) Wir danken Herrn Prof. Dr. W. Lorenz, daß er uns die Untersuchungen in der Universitätsklinik für Strahlentherapie und Nuclearmedizin Frankfurt/M. ermöglichte, sowie Herrn Dr. S. Gursky für seine freundliche Hilfe bei den Messungen.

Ergebnisse

1 Erzeugung der Verdünnungsanämie mit Macrodex

Macrodex eignet sich wegen seiner relativ langen Verweildauer in der Blutbahn (2) gut zur Erzeugung einer Verdünnungsanämie. Wir infundierten meist 2 × täglich 18 ml/kg über 7 Tage (insgesamt 250 ml/kg). Der größte Teil des Plasmaexpanders wird bei derartig gehäuften Gaben wieder ausgeschieden oder aber im extravasalen

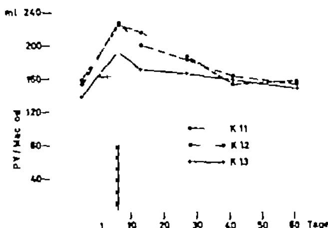


Abb. 1 Änderungen des Plasmavolumens bei 3 Kaninchen nach 6tägiger Macrodex Infusion.

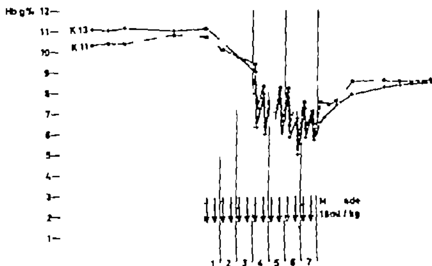


Abb. 2 Hämoglobinabfall bei Messung jeweils vor und 30 min nach Macrodex Infusion.

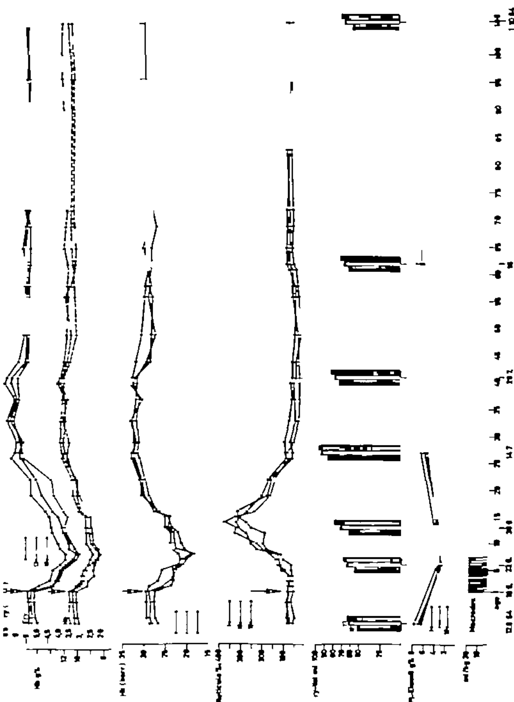


Abb. 3 Die Änderungen von Erythrocytenkonzentration und -gesamtmenge, Reticulocytenzahl und Plasmaeiweiß im Verlauf der Verdünnungsanämie bei 3 Kaninchen.

Tabelle I

Ausmaß der Verdünnungsanämie, maximale Retikulozytose und reaktl Polyglobulie bei 12 Kaninchen.

Tier Nr	Ausgangswerte Hb g%	Ery Mill.	Hk	Ges.-EW g%	»Maximale Anämie« Hb g%	Ery Mill.	Hk %	Ges.-EW g%
1	10,3	4,6	32	5,65	6,8	3,2	24	3,85
2	10 6	4,8	32	6,45	6,3	3,3	22	3,85
3	11,2	5,5	35	6,0	5,5	2,9	19	3,25
4	11 0	4,8	33	6,05	6,4	2,8	20	3,9
5	11,8	5 7	36	5,75	6,5	3,3	22	4,0
6	10,6	4,9	32	6,4	6,4	3,1	19	4 1
8	11 7	5,3	36	—	7,2	3,4	24	—
9	12,3	5,5	36	—	7,5	3,0	25	—
10	11,9	5,6	36	—	6,2	3,0	22	—
11	10,5	5 4	32	6,6	7,5	3,6	24	3,1
12	9,9	5,1	30	5,55	6,9	3,2	21	3,2
13	11 1	3,3	32	6,05	6,5	3,0	20	3,2

Raum abgelagert und strömt erst in der Folgezeit allmählich wieder in die Gefäße zurück. Bei 2 Tieren, die unter der Infusionsbehandlung starben, fanden wir erhebliche Pleuraergüsse und Ascites Aus Abb 1 geht hervor daß nach Infusion von insgesamt 900 ml das PV nur um 65 ml im Mittel anstieg

Die Blutkontrollen wurden täglich jeweils vor den Infusionen durchgeführt. Um den täglich auftretenden Tiefpunkt der Anämie zu erfassen, haben wir bei 2 Tieren die Hb-Werte jeweils vor und 30 min nach Ende der Infusionen bestimmt (Abb 2) Jede Infusion des Plasmaexpanders führt zu einem Hb-Abfall von 1–2 g / Die Hb-Konzentration steigt dann in den folgenden Stunden wieder an ohne den vorherigen Wert wieder zu erreichen.

Die Infusionsbehandlung wurde beendet, wenn die Anämie den gewünschten Schweregrad erreicht hatte und bei weiterer Volumenerhöhung eine deletäre Kreislaufüberlastung zu erwarten gewesen wäre

2 *Blutveränderungen bei Verdünnungsanämie*

In Abb 3 ist der Verlauf von Hb Erythrozytenzahl und Hk während der Verdünnungsanämie bei 3 Tieren dargestellt Die übrigen 9 Tiere verhielten sich völlig gleichsinnig (Tabelle I) Etwa am 6 bis 9 Tag nach Beginn der Infusionsbehandlung war der gewünschte Tiefpunkt der Anämie erreicht der Mittelwert des Hb auf 6 6g / abgefallen.

Tabelle I (Fortsetzung)

[illegible]	Reticulocyten Anstieg Tag	max. Retic.-Anstieg Tag	‰	d. Vorw.	Maximale Polyglobulie Tag	Hb g%	Ery. Mill.	Hk
63	4	(9.)	298	458	am 9. Tag gestorben			
64	5.	13.	215	336	nicht untersucht			
84	5.	17	322	383	nicht untersucht			
70	6.	11	222	317	nicht untersucht			
63	5	11	285	419	nicht untersucht			
70	5.	11	196	280	33	11,2	6,2	34
72	7	13.	300	417	35.	13,0	6,2	38
50	5.	11	269	528	40.	13,9	6,3	42
74	7	12.	356	481	38.	13,0	6,2	39
59	5.	16.	321	545	40.	12,5	6,4	37
73	5.	13.	311	427	40.	12,0	6,1	36
72	5.	14.	376	522	33.	11,9	6,0	36

Die Blutverdünnung löst gesetzmäßig eine Aktivierung der Erythropoese aus, erkennbar an einer erheblichen Reticulocytose. Diese begann meist am 5. (4. bis 7.) Tag nach Infusionsbeginn und erreichte am 11. bis 17. Tag ihr Maximum, durchschnittlich 6 (4 bis 10) Tage nach Beendigung der Infusionen. Setzt man den Ausgangswert der Retic. (‰) gleich 100 %, dann betrug der maximale Retic.-Anstieg 280 bis 545 %, im Mittel 425 % dieses Ausgangswertes (Tabelle I). Diese enorme Ausschwemmung von Retic. geht mit einer sehr deutlichen Linksverschiebung einher, also einer Vermehrung der Knäuel- und Netzformen (Abb. 4). Die Retic.-Zahl erreicht den Ausgangswert nach unterschiedlicher Zeit (16 bis 32 Tage nach Beendigung der Infusionen, im Mittel nach 21 Tagen). Auf Einzelheiten der Retic.-Kurve wird weiter unten eingegangen.

Nach Beendigung der Infusionen bleiben Hb-, Ery- und Hk-Werte in den folgenden Tagen niedrig. Der Zeitpunkt, zu dem die *Blutwerte zum Vor-Wert zurückgekehrt sind, hängt von der Geschwindigkeit ab*, mit der das im extravasalen Raum eingelagerte Macrodex in die Blutbahn zurückströmt. Im Anschluß an die Verdünnungsanämie kommt es infolge der Aktivierung der Erythropoese zu einer vorübergehenden Polyglobulie (Tabelle I, letzte Spalte). Das Maximum dieser Polyglobulie war zwischen dem 27. und 33. Tag nach Infusionsende erreicht (Anstieg des Hb von 11,1g% auf 12,5g%, der Erythrocyten von 5,3 auf 6,2 Mill./mm³).



Abb. 4 Vermehrung der unreifen Reticulozyten auf dem Höhepunkt der Reticulozytose

Gesetzmäßig sinkt die Retic.-Zahl in dieser 2. Phase erheblich unter den Ausgangswert ab. Der Tiefpunkt dieser negativen Retic.-Bewegung ist nach 34 bis 49 (im Mittel nach 40) Tagen erreicht, die Retic.-Werte betragen zu diesem Zeitpunkt 39 % des Ausgangswertes (vgl. Tabelle III, Zeitpunkt V).

Bei 4 Tieren wurden die Blutkontrollen fortgesetzt, bis alle Blutwerte zur Norm zurückgekehrt waren. Dies war für Hb und Erythrozytenzahl zwischen dem 50. und 65., für die Retic.-Zahl zwischen dem 60. und 90. Tag nach Ende der Infusionsbehandlung der Fall. Es dauert also 2 bis 3 Monate, bis die Störung des steady state völlig ausgeglichen ist, die durch eine 7tägige Infusionsbehandlung mit dem Plasmaexpander hervorgerufen wurde.

Parallel zur Abnahme der Erythrozyten kommt es unter der Behandlung mit dem Plasmaexpander auch zu einer „Verdünnung" der Plasmaeiweißkörper (Abb. 3, Tabelle I). Der Ausgangswert ist 8–24 Tage nach Beendigung der Macrodexinfusionen wieder erreicht, es kommt dann zu einem weiteren Anstieg des Plasmaeiweißes, der längere Zeit anhält (64), analog der reaktiven Polyglobulie.

In einer gesonderten Mitteilung werden wir auf diese Eiweißverschiebungen eingehen, insbesondere auch im Hinblick auf das zirkulierende Totaleiweiß.

Tabelle II

tive Erythrozytenzahl (pro cmm) absolute Menge (EV) und Total-Erythrozytenzahl in der 2. Phase der Verdünnungsanämie.

	Datum	Hb g%	Ery./cmm	Hk %	EV ml	PV ml	BV ml	Ery Total 10^{9}	% des Ausg.-Wertes Ery./cmm	EV	Total-Ery
[illegible]	12.6.64	10,5	5,4	29,5	63	157	220	1188			
	14.7 64	10,8	5,95	32,4	94	186	280	1660	110	149	140
[illegible]	*12.6.64*	*9,9*	*5,1*	*27,6*	*51*	*139*	*190*	*969*			
	14 7 64	11,2	5,83	32,4	84	166	250	1457	115	165	150
[illegible]	12.6.64	11,1	5,3	29,5	61	153	214	1134			
	14 7 64	11 6	5,6	32,4	93	183	276	1546	106	153	156

3 Erythrozytenvolumen (EV) und Total Reticulozytenzahl

In Abb 3 sind die Werte des EV im Verlauf der Verdünnungsanämie den Konzentrationswerten (Hb Ery Hk) gegenüber gestellt. Anhand der absoluten EV Werte ist ersichtlich, daß es sich in der Tat um eine reine Blutverdünnung handelt. Denn während die Konzentrationswerte stark abfallen, bleibt das EV unverändert und steigt infolge des inzwischen eingetretenen Retic. Anstiegs an. Der maximale Wert des EV wird dann erreicht, wenn die Reticulozytose gerade abgeklungen ist und die Konzentrationswerte gering über den Ausgangswert angestiegen sind. Da aber das PV zu diesem Zeitpunkt immer noch deutlich vergrößert ist (20 Tage nach Beendigung der Macrodex Infusionen!) ist – bezogen auf den Ausgangswert – die Erhöhung des EV größer als es der Erhöhung der Ery Zahl/mm entspricht (Tabelle II) Zu diesem Zeitpunkt besteht also eine Polyämie, bei der die EV Erhöhung infolge der gleichzeitig bestehenden PV Erhöhung nicht zu erkennen ist und nur durch eine BV Bestimmung erfaßt werden kann.

Infolge der nun einsetzenden PV Abnahme (Ausscheidung des Macrodex) steigen Hb und Erythrozytenzahl noch weiter an bis zu den Werten, die in Tabelle I (letzte Spalte) angegeben sind.

Aus Tabelle II geht außerdem hervor daß die Zunahme des EV bezogen auf den Ausgangswert etwas größer ist als die der Total-Erythrozytenzahl. Dies dürfte darauf beruhen, daß das mittlere Volumen des einzelnen Erythrozyten (MCV) infolge der vorangegangenen Reticulozytose größer als normalerweise ist. Auf die Änderungen des Erythrozyten-Einzelvolumens im Verlauf der Verdünnungsanämie werden wir in einer späteren Mitteilung eingehen.

Die absolute Vermehrung der Erythrozyten (EV) hält außerordentlich lange an, so daß selbst bei der Messung 106 Tage nach



Abb. 4 Vermehrung der unreifen Reticulocyten auf dem Höhepunkt der Reticulocytose

Gesetzmäßig sinkt die Retic. Zahl in dieser 2. Phase erheblich unter den Ausgangswert ab. Der Tiefpunkt dieser negativen Retic. Bewegung ist nach 34 bis 49 (im Mittel nach 40) Tagen erreicht, die Retic. Werte betragen zu diesem Zeitpunkt 39 des Ausgangswertes (vgl. Tabelle III, Zeitpunkt V).

Bei 4 Tieren wurden die Blutkontrollen fortgesetzt, bis alle Blutwerte zur Norm zurückgekehrt waren. Dies war für Hb und Erythrozytenzahl zwischen dem 50. und 65., für die Retic. Zahl zwischen dem 60. und 90. Tag nach Ende der Infusionsbehandlung der Fall. Es dauert also 2 bis 3 Monate, bis die Störung des steady state völlig ausgeglichen ist, die durch eine 7tägige Infusionsbehandlung mit dem Plasmaexpander hervorgerufen wurde.

Parallel zur Abnahme der Erythrocyten kommt es unter der Behandlung mit dem Plasmaexpander auch zu einer Verdünnung der Plasmaeiweißkörper (Abb. 3, Tabelle I). Der Ausgangswert ist 8–21 Tage nach Beendigung der Macrodexinfusionen wieder erreicht, es kommt dann zu einem weiteren Anstieg des Plasmaeiweißes, der längere Zeit anhält (A), analog der reaktiven Polyglobulie.

In einer gesonderten Mitteilung werden wir auf diese Eiweißveränderungen eingehen, insbesondere auch im Hinblick auf das zirkulierende Totaleiweiß.

Tabelle III

Reticulocytenbewegung im Verlauf der Verdünnungsanämie.

Datum	Zeitpunkt	Retic. ‰	% des Vor-wertes	Retic./mm³	% des Vor-wertes	Total-Retic. 10^9	% d. Vor-wertes
28. 3.	I	72	100	383000	100	735	100
7. 4.	II	77	107	263000	68	—	
17. 4.	III	310	431	1120000	291	—	
29. 4.	IV	70	97	356000	93	—	
22. 5.	V	32	44	195000	51	390	53
8. 4.	I	50	100	274000	100	563	100
13. 4.	II	53	106	249000	91	—	
20. 4.	III	269	538	892000	326	—	
5. 5.	IV	54	108	278000	102	718	127
2. 6.	V	19	38	113000	41	269	47
28. 3.	I	74	100	412000	100	702	100
8. 4.	II	88	118	262000	64	—	
13. 4.	III	356	481	1350000	328	—	
25. 4.	IV	75	101	387000	94	—	
22. 5.	V	32	43	189000	46	355	51
12. 6.	I	59	100	318000	100	726	100
23. 6.	II	72	122	274000	86	795	110
30. 6.	III	253	428	1088000	342	3190	440
3. 7.	(III)	321	545	1457000	457		
14. 7.	IV	58	98	345000	109	966	133
5. 8.	V	19	32	103000	33	—	
18. 8.	VI	44	75	231000	73	520	72
1. 10.	VII	66	112	350000	110	750	103
12. 6.	I	73	100	376000	100	697	100
23. 6.	II	108	147	364000	97	898	129
30. 6.	III	311	427	1230000	327	2850	410
14. 7.	IV	81	111	472000	126	1180	169
28. 7.	V	46	63	273000	74	632	90
5. 8.	(V)	34	47	181000	48		
18. 8.	VI	68	93	352000	94	735	105
1. 10.	VII	75	103	384000	102	753	108
12. 6.	I	72	100	381000	100	805	100
23. 6.	*II*	*118*	*164*	*400000*	*105*	*1160*	*144*
5) 30. 6.	III	310	431	1200000	315	3260	405
1. 7.	(III)	376	522				
14. 7.	IV	74	103	415000	109	1145	143
28. 7.	V	20	28	115000	30	275	34
18. 8.	VI	59	82	307000	81	670	83
1. 10.	VII	68	95	350000	92	740	92

...ehpunkte: I = vor Versuchsbeginn, II = vor oder zu Beginn der Reticulocytose, III = ...er Reticulocytenanstieg, IV = Rückgang der Reticulocytenzahl (‰) zum Ausgangswert, ...punkt der Reticulocytenbewegung, VI = Wiederanstieg der Reticulocyten zum Ausgangs- ...I = Spätwert bei Versuchsende. Bei K 8–10 wurde die Total-Reticulocytenzahl nur 2× ...t, bei K 11–13 bei Zeitpunkt I–VII.

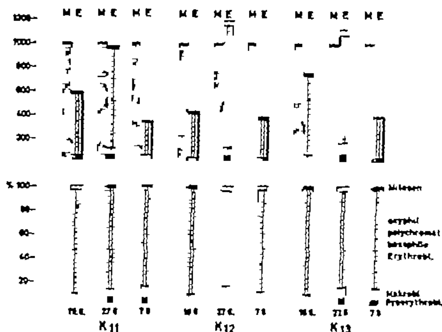


Abb. 6. M/E-Index und relative Verteilung der Reifestadien der Erythropoese. 19. 6. = vor dem Versuch. 27. 6. = Höhepunkt der Reticulocytose. 7. 8. = Hemmung der Erythropoese.

erhöht, ebenso die ‰ Retic., während Retic./mm³ noch normal bzw. erniedrigt sind. Auch auf dem Höhepunkt der Reticulocytose (= Zeitpunkt III) ist die Zahl der Retic./mm³ wesentlich niedriger als es den tatsächlichen Verhältnissen entspricht; die ‰ Retic. Zahl stimmt jedoch gut mit der Total Retic. Zahl überein. Zum Zeitpunkt IV des Versuchs sind die Werte in ‰ und pro mm³ etwa zum Ausgangswert zurückgekehrt. EV und BV haben inzwischen ihr Maximum erreicht, so daß die Total-Retic. Zahl noch deutlich erhöht ist.

Abb. 5 und Tabelle III zeigen überdies eindrucksvoll, wie stark in der 2. Phase im Stadium der reaktiven Polyglobulie, die Retic.-Einschwemmung aus dem Knochenmark eingeschränkt ist.

Zusammenfassend ergibt sich, daß die Retic. Zahl nur bei Berücksichtigung der Blutmenge einen wirklichen Rückschluß auf die Erythrozytenproduktion erlaubt. Die Angabe in ‰ der Erythrozyten kommt den Total Retic. Werten näher als die Angabe pro mm³.

4 Knochenmarksbefunde

Bei 3 Tieren wurde das Knochenmark vor Versuchsbeginn, unmittelbar vor dem Höhepunkt der Reticulozytose und zum Zeitpunkt der Retic. Depression untersucht und die Relation von Granulo- zu Erythropoese (M/E-Index) ermittelt. Innerhalb der Erythropoese wurde differenziert in Proerythroblasten (Kernklasse K 2) Makroblasten (K 1) basophile, polychromatische und oxyphile Normoblasten (K 1/2, K 1/4 K 1/8) zusammengefaßt Mitosen. Im oberen Teil von Abb 6 ist die Aktivierung der Erythropoese als Folge der Blutverdünnung und ihre Hemmung als Folge der reaktiven Polyglobulie an dem veränderten M/E-Index zu erkennen. Der untere Teil der Abb gibt den relativen Anteil der einzelnen Reifestadien der Erythropoese wieder Bemerkenswert ist, daß die Relation der Proerythroblasten zu den übrigen Erythroblasten etwa konstant bleibt, wie dies von Schwarz (50) und Wicker (66) unter normalen und auch unter pathologischen Bedingungen gefunden wurde.

5 Bestimmung der Lebenszeit der Erythrozyten

Um mit Sicherheit annehmen zu können, daß es sich bei der Aktivierung der Erythropoese allein um die Auswirkung der Blutverdünnung handelt, war auszuschließen, daß es zu einer Hämolyse infolge der hohen Macrodexkonzentrationen im Blut kommt, zumal von Norlander *et al.* (40) bei Dextrangaben beim Menschen eine vermehrte Zelldestruktion diskutiert wird.

Die Werte des EV das zunächst konstant bleibt und im weiteren Verlauf deutlich zunimmt (s. o.) lassen eine stärkere Zellzerstörung bereits weitgehend ausschließen. Der sichere Beweis dafür daß es unter den Macrodex Infusionen zu keiner Hämolyse kommt, ist durch die unveränderte Lebenszeit (Lz.) der Erythrozyten gegeben. Lz. Bestimmungen chrommarkierter Erythrozyten wurden bei 5 Tieren durchgeführt. Bei 3 Tieren begannen wir mit den Infusionen 6 Tage nach der Chromierung, bei 2 Tieren bereits am folgenden Tag (Abb. 7) Die tägliche Abbaurate der Erythrozyten bleibt unverändert während der Blutverdünnung die an dem Absinken des Hk zu erkennen ist. Damit ist eine vermehrte Hämolyse als Ursache der Aktivierung der Erythropoese ausgeschlossen.

Diese Messungen können nur im Erythrozytensediment durchgeführt werden, da bei einer Messung im Vollblut infolge der gleichzeitigen Blutverdünnung (und damit

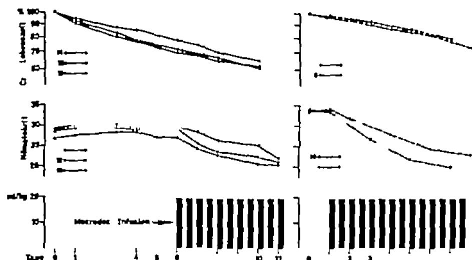


Abb. 7 Erythrozyten-Lebenszeit während der Infusionen von Macrodex.

Abnahme der Ery/Raumeinheit) eine stärkere Abnahme der Aktivität und damit eine scheinbare Lz.-Verkürzung resultieren würde. Die Lz.-Kurven sind auch nur solange verwertbar, wie die Retic. noch nicht angestiegen sind. Mit Beginn der Reticulozytose wird der relative Anteil markierter Zellen durch die vermehrte Einschwemmung unmarkierter, d. h. nach der Chromierung gebildeter Zellen vermindert. Auch dadurch wird die Lz. der markierten Erythrozyten scheinbar verkürzt. Die Beziehung zwischen Retic.-Zahl und Cr^{51}-Lz. *der Erythrozyten wird aus Abb. 8 deutlich: die erste Lz.* Bestimmung (ausgezogene Kurve) wurde vor Versuchsbeginn, die zweite (gestrichelt) bei Anstieg der Retic. vorgenommen. Die zweite Kurve zeigt einen Knick nach unten, d. h. eine scheinbare Verkürzung der Lz., sobald die Retic. stärker ansteigen. Von Stohlman (37) wurde empfohlen, bei ansteigender Erythrozytenzahl (also z. B. während einer Retic. Krise) die Lz.-Bestimmung im Vollblut durchzuführen. Dies ist zweifellos besser als die Bestimmung im Erythrozytensediment, kann aber nur dann zu richtigen Ergebnissen führen, wenn das BV konstant bleibt, d. h. wenn das PV in dem gleichen Ausmaß abnimmt, wie das EV zunimmt. Bei der experimentellen Verdünnungsanämie ist dies nicht der Fall, bei der Regeneration chronischer Anämien nicht immer (25).

Weiterhin war die Bestimmung der Lz. jener Erythrozyten von Interesse, die in der Phase der starken Aktivierung der Erythropoese gebildet *worden waren. Da in dieser Phase kein Fließgleichgewicht* besteht (Schwankungen der Erythrozytenneubildung und des PV) kann eine solche Lz. Bestimmung nicht beim Versuchstier selbst erfolgen, sondern nur nach Übertragung auf ein Empfängertier. Etwa auf dem Höhepunkt der Reticulozytose wurde bei 8 Tieren Blut entnommen, nach weiterer Anreicherung jugendlicher Erythrozyten (s. Methoden) mit Cr^{51} markiert und auf unbehandelte Empfängertiere übertragen. Bei einem dieser Tiere

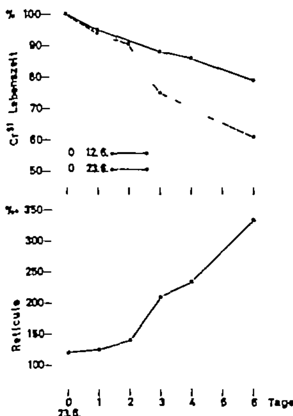


Abb. 8. Die Beziehung zwischen der Reticulocytenzahl und dem Aktivitätsabfall nach Markierung mit Cr^{51} (Messung im Erythrocytensediment)

kam es am 8. Tag infolge Bildung von Isoantikörpern zu einem rapiden Abfall der Aktivität auf Null. Die Lebenszeitkurven der beiden anderen Tiere sind in Abb 9 dargestellt, im Vergleich zu den Lz. Kurven, die bei den Versuchstieren selbst vor Versuchsbeginn gewonnen worden waren.

Die Lz. Kurve der übertragenen Zellen verläuft flacher entsprechend dem größeren Anteil jüngerer Zellen in dieser Population. Ihre Cr Halbwertszeit betrug 19 bzw 22 Tage, im Vergleich zu 16 bzw 17 Tage vorher Bei dieser nur relativ geringfügigen Verlängerung der mittleren Lz. ist aber zu berücksichtigen, daß es sich bei diesen Erythrozyten zum Zeitpunkt der Reticulocytose nicht um eine reine «Kohorte» (14) handelt, d. h. um junge, zur gleichen Zeit im Knochenmark gebildete Zellen, sondern lediglich

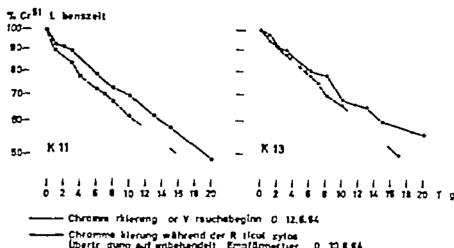


Abb. 9. Lebenszeit der Erythrozyten vor Versuchsbeginn und auf dem Höhepunkt der Reticulozytose. – Aus methodischen Gründen wurde die Vors-Kurve nur bis zum 10. Tag gemessen und dann bis zur 50%-Achse verlängert.

Tabelle IV

Lebenszeit radiochrommarkierter Erythrozyten in verschiedenen Phasen der Verdünnungsanämie (t/2).

Zeitpunkt		K 11	K 12	K 13
12. 6.	Vor Versuchsbeginn	~ 16 T	~ 16 T	~ 17 T
30. 6.	Reticulozytose, Übertragung auf Empfängertiere	19 T	—	~ 22 T
28. 7	*Hemmung der Erythropoese,* Reticulozyten vermindert	~ 17 T	~ 17 T	~ 22 T

Die Messungen konnten aus methodischen Gründen meist nicht ganz bis zur t/2 durchgeführt werden. Die Cr[51]-Halbwertszeit wurde dann durch Verlängern der Lebenszeitkurve bis zur 50%-Achse ermittelt.

um eine Anreicherung jüngerer Erythrozyten neben älteren Zellen, die sich schon vor der Reticulozytose im Blut befanden.

Orientierende Lz. Bestimmungen – mit weniger Meßpunkten – wurden von den gleichen Tieren auch im weiteren Verlauf durchgeführt. Die Ergebnisse sind in Tabelle IV zusammengestellt.

Die scheinbare Verlängerung von t/2 zur Zeit der Hemmung der Erythropoese hat den gleichen Grund wie – im umgekehrten Falle – die scheinbare Verkürzung während des Retic.-Anstiegs (Abb. 8): da der Zellnachschub geringer ist als die (normal weitergehende) Destruktion, verschiebt sich die Relation von markierten zu unmarkierten Erythrozyten weniger, als wenn Produktion und Destruktion im Gleichgewicht stehen.

Diskussion

I. Die Regulation der Erythropoese bei der Verdünnungsanämie

Die experimentelle Verdünnungsanämie hat unter den hämatologischen Krankheitsbildern kein typisches Analogon. Bei vielen Anämien spielt zwar eine Verdünnungskomponente eine gravierende Rolle, hierbei handelt es sich dann aber um eine primäre Abnahme des EV, die durch eine PV-Zunahme volumenmäßig kompensiert bzw. häufig überkompensiert wird (5, 33). Eine primäre PV-Vermehrung, die über eine Blutverdünnung eine verstärkte Erythropoese auslöst, dürfte in einzelnen Fällen von Lebercirrhose (37), sicher aber bei der dekompensierten Herzinsuffizienz von Bedeutung sein (31, 55). In solchen Fällen fand HEDLUND neben einer erheblichen PV-Vermehrung eine hyperplastische Erythropoese, Retic.- und EV-Vermehrung. Es handelt sich hierbei aber auch nicht ausschließlich um die stimulierende Wirkung der PV-Vermehrung auf das Knochenmark, da außerdem die kardial bedingte Gewebshypoxie erythropoetisch wirksam ist.

Die experimentelle Erzeugung einer länger anhaltenden reinen Scheinanämie setzt die Anwendung einer Substanz mit möglichst langer Verweildauer in der Blutbahn und einer guten Verträglichkeit auch in hoher Konzentration voraus. Diese Bedingungen sind bei Macrodex erfüllt (Übersicht bei 51), von dem sich nach einmaliger Infusion beim Menschen nach 8 Std. noch 50% und mehr (2, 28), nach 24 Std. noch etwa 30% in der Zirkulation befinden (28, 49, 64). Bei Infusion so großer Mengen, wie sie zur Erzeugung einer Verdünnungsanämie erforderlich sind, verschwindet Macrodex allerdings schneller aus der Blutbahn (Ausscheidung über Niere und Magen-Darm, Abwanderung in den extravasalen Raum, Abbau), wie aus dem Mißverhältnis von zugeführter Macrodexmenge und Anstieg des PV hervorgeht (Abb. 1). Eine die Erythrozyten direkt schädigende Einwirkung von Macrodex, auch in Konzentrationen, die den Hk erheblich senken, ist nicht nachweisbar. Dies geht aus dem unveränderten Aktivitätsabfall nach Markierung der Erythrozyten mit Cr^{51} (Abb. 7) und der Zunahme des EV im Verlauf der Verdünnungsanämie hervor.

Über die Wirkung protrahierter Gaben von Plasmaexpandern auf die Erythropoese liegen nur wenige Angaben vor. Die klinische Indikation dieser Stoffe ist die Auffüllung des Kreislaufs nach Blutverlusten, eine fortlaufende Anwendung, die zu einer länger anhal-

tenden Blutverdünnung führen könnte, verbietet sich deshalb. Zudem wird die Erythropoese in solchen Fällen ohnehin durch den vorausgegangenen Blutverlust aktiviert.

Lediglich von GASANOV (zit. 20) und GOLDMAN (26) wurde über eine Stimulierung der Erythropoese nach Infusionen mit Polyglucin, einem russischen Dextran-Präparat mit etwa dem gleichen mittleren Molekulargewicht wie Macrodex, berichtet. ERSLEV (18) dagegen konnte nach 4tägiger Infusion von Dextran bei Kaninchen keine Erythropoesesteigerung (gemessen an Retic. Zahl und M/E-Index im Knochenmark) feststellen. Dieser Befund, der bisher bei allen Überlegungen zur Regulation der Erythropoese eine wichtige Rolle gespielt hat, dürfte darauf zurückzuführen sein, daß ERSLEV seine Blutkontrollen nicht lange genug ausdehnte. Im Gegensatz zu den von ihm durchgeführten Aderlaßversuchen, bei denen die Erythrozytenverminderung plötzlich eintritt und einen Retic. Anstieg bereits nach 48 Std. auslöst, tritt die Dilutionsanämie allmählich ein und erreicht den gleichen Schweregrad erst am 4 Infusionstag. ERSLEV führte aber seine Knochenmarkskontrolle bereits am 5 Tag durch und verfolgte den Retic. Verlauf nur bis zum 8 Tag

Der Verdünnungsanämie kommt beim Problem der Erythrozytenregulation insofern grundsätzliche Bedeutung zu als nach unseren Vorstellungen die Sauerstoffspannung im Gewebe an den noch unbekannten hypoxieempfindlichen Rezeptoren das erste Glied innerhalb der humoralen Steuerung der Erythropoese ist. Die Menge des im Gewebe verfügbaren Sauerstoffs hängt nun in erster Linie von der Erythrozyten(Hämoglobin)-Konzentration ab Sinkt sie, dann kann dies – neben einer vermehrten Sauerstoffausnutzung (54) – nur in geringem Ausmaß durch Erhöhung des Herzminutenvolumens und der Umlaufgeschwindigkeit des Blutes ausgeglichen werden. *Zur völligen Kompensation der anämiebedingten Hypoxie reichen diese Kreislaufveränderungen aber nicht aus* (32, 35) im Gegensatz zu der Annahme von ERSLEV mit der er seine Befunde zu erklären versuchte.

Bei der Verdünnungsanämie liegt zudem sicher die Situation einer Herzinsuffizienz als Folge der rasch erzeugten Hypervolämie vor die mit einer Abnahme des Minutenvolumens und Verlangsamung der Strömungsgeschwindigkeit einhergeht (32) Bei 3 Kaninchen haben auf dem Höhepunkt der Verdünnungsanämie die Kreislaufzeit gemessen (Injektion von *Coomassieblau in ein Ohr*

Ohreinheit am andern Ohr) Sie war gegenüber der Voruntersuchung unverändert. Bei der Verdünnungsanämie gleichen sich also die Beschleunigung der Zirkulation infolge Anämie und die Verlangsamung infolge Herzinsuffizienz und Hypervolämie aus.

Da die kreislaufdynamische Anpassung nur eine untergeordnete Rolle spielt, stellt die Erythrozyten bzw Hämoglobinkonzentration den entscheidenden Parameter für die Sauerstoffspannung im Gewebe und damit für die Regulation der Erythropoese dar

Unsere Versuche zeigen, daß die absolute Größe des EV ohne Bedeutung für diese Regulation ist. Sie sprechen damit gegen die von Brecher und Stohlman (12) aufgestellte Hemmfaktorentheorie. Dieser Theorie zufolge enthält der alternde Erythrozyt einen Hemmfaktor für das Knochenmark. Eine Verminderung der Hemmfaktoren (Blutverlust, Hämolyse) führt zur Erythrozytenproduktion, eine Zufuhr von Hemmfaktoren (Transfusion) zu einer Knochenmarkshemmung So bestechend diese Theorie zur Erklärung z. B. des völlig kompensierten hämolytischen Syndroms (bei dem eine Hypoxie keine Rolle spielt) sein mag, bei der Verdünnungsanämie tritt die Stimulierung der Erythropoese auf, ohne daß ein «Hemmfaktorenverlust» vorausgegangen wäre. Denn das EV bleibt unverändert.

Die erhebliche Mehrproduktion von Erythrozyten ohne gleichzeitigen Zellverlust muß zwangsläufig zu einer vorübergehenden Polyglobie führen. Diese kommt in der Zunahme des absoluten EV deutlicher zum Ausdruck als im Anstieg der Konzentrationswerte, weil die noch immer bestehende PV Vermehrung sich im Sinne einer Verdünnung auswirkt.

Für die Rückkehr von diesen überhöhten Werten zur Norm sind 2 Möglichkeiten denkbar entweder tritt eine vorübergehende Hemmung der Zellneubildung auf, oder aber die überschüssig gebildeten Erythrozyten werden vorzeitig abgebaut.

Für diese zweite Möglichkeit, die Verkürzung der Lebensdauer der neugebildeten Zellen, sind klinische und experimentelle Beispiele bekannt.

Eine Reihe von Autoren konnte zeigen, daß bei starker Aktivierung der Erythropoese nach Hämolyse, Blutverlust und Erythropoetingaben sowie nach Behandlung von Eisenmangelanämien besonders große Erythrozyten (bzw Retic.) mit verkürzter Lebensdauer ins Blut gegangen (7 10, 11 13, 14 30, 38, 39 58, 59, 61 63) Die Autoren nehmen an, daß diese Retic. nach beschleunigter Reifung unter Überspringung von Reifestellungen und vorzeitiger Entkernung ausgeschwemmt werden. Der Grad der Makrozytose und der Lebenszeitverkürzung ist dabei von der Intensität der vorausgegangenen Stimulierung abhängig (59) Es ist jedoch nicht ihre Größe, die diese Zellen vorzeitig der Hämolyse anheimfallen läßt, da auch bei splenektomierten Tieren die

gleiche Lebenszeitverkürzung beobachtet (60). Man muß vielmehr einen Defekt dieser überstürzt gebildeten Zellen annehmen. Im Knochenmark wird eine Vermehrung der unreifen Vorstufen der Erythropoese gefunden (55).

Dieser „Panikmechanismus" (59) der Erythropoese wirkt sich scheinbar wie eine sinnvolle Steuerung aus, da zunächst viele Zellen produziert werden und der Ausgangswert ohne stärkere Gegenschwankungen wieder erreicht wird. Um eine echte Regulation handelt es sich dabei aber nicht.

Es ist außerdem darauf hinzuweisen, daß dieser Befund einer verkürzten Lebensdauer überstürzt gebildeter Erythrozyten von anderen Autoren (1, 19) nicht bestätigt werden konnte.

Bei der Verdünnungsanämie liegen die Verhältnisse nun aber grundsätzlich anders, d. h. es handelt sich um eine echte Regulation, die auf die scheinbare Anämie mit einer Aktivierung der Erythropoese und Ausschwemmung von Erythrozyten mit normaler Lebenszeit, auf die reaktive Polyglobulie dagegen mit einer Markhemmung antwortet. Unsere Befunde unterscheiden sich deshalb in wichtigen Punkten von denen, die bei dem oben skizzierten „Panik-Mechanismus" erhoben wurden.

Die Relation der einzelnen Reifestadien der Erythropoese, insbesondere die der Proerythroblasten zu den übrigen Erythroblasten bleibt unter der Stimulierung unverändert (Abb. 6). Der Retic.-Anstieg setzt zumeist erst am 5. (4. bis 7.) Tag ein (Tabelle I), nicht schon nach 48 Stunden wie in den Experimenten von STOHLMAN et al. (59). Die Lebensdauer der neu gebildeten Zellen ist nicht verkürzt (Abb. 7). Die Folge dieser Mehrbildung vollwertiger Erythrozyten ist eine starke Vermehrung des EV (Tabelle II) und eine Polyglobulie. Diese löst eine Hemmung der Erythropoese aus, erkennbar an dem verminderten Anteil der Erythroblasten im Mark (Abb. 6) und dem Absinken der Retic. unter den Ausgangswert (Tabelle III, Abb. 5).

Die gleiche Regulation, die somit allein über eine Änderung der Knochenmarksaktivität wirksam wird, findet sich auch bei der Höhenanpassung, sowohl in der Phase der Mehrproduktion von Erythrozyten (35) als auch nach Rückführung Höhenangepaßter in normale Sauerstoffverhältnisse (45). Ändert sich jeweils die Zellproduktion, nicht aber die Destruktionsrate. Ebenso wird auch nach einer Transfusions-Polyglobulie die normale Zellkonzentration nicht durch eine vermehrte Hämolyse, sondern durch eine vorübergehende Knochenmarkshemmung erreicht (9), wahrscheinlich über einen humoralen Inhibitor (36, 46). Eine sichere Erklärung der unterschiedlichen Reaktion der Erythropoese auf die Stimulierung durch Aderlaß, Hämolyse und Erythroproteingaben einerseits und

der Verdünnungsanämie andererseits kann vorerst nicht gegeben werden. Es ist zu vermuten, daß die unterschiedliche Geschwindigkeit, mit der die Anämie eintritt, eine wichtige Rolle spielt. Bei plötzlich einsetzender Anämie würde dann der «Panik Mechanismus» der Ausschwemmung kurzlebiger Makrozyten, bei allmählich einsetzender Anämie die geregelte Bildung normallebiger Erythrozyten einsetzen.

II Die Verdünnungsanämie als Modell

Die experimentelle Verdünnungsanämie als Modell ist geeignet, die engen Beziehungen aufzuzeigen, die zwischen Gesamtblutmenge und Zellkonzentration bestehen. Hb-Wert, Erythrozytenzahl und Retic. Zahl geben uns nur dann einen verläßlichen Hinweis auf die Knochenmarksleistung, wenn das Blutvolumen keine größeren Abweichungen von der Norm zeigt. Steigt das PV – z. B im Rahmen einer Anämie – an, dann ist die Gesamtmenge zirkulierender Erythrozyten größer als es nach der Erythrozytenzahl scheint. Diese Konstellation ist geläufig z. B bei der Schwangerschaftshydrämie.

Weniger in Betracht gezogen wird zumeist, daß bei der Bewertung der Retic. Zahl, einem der wichtigsten Parameter zur quantitativen Erfassung der Erythrozytenproduktion, die Gesamtblutmenge ebenfalls eine Rolle spielen kann. Nicht nur die relative Retic. Zahl (angegeben in % oder ‰) sondern auch die sog «absolute» Zahl (Retic./mm³ oder die für den Hk Wert korrigierte Retic. Zahl (24) erhält unter diesem Gesichtspunkt u. U einen nur relativen Wert.

Im Extremfall der Verdünnungsanämie mit ihren großen Änderungen des Blutvolumens wird dies evident hier kommt es zu einer erheblichen Diskrepanz zwischen der absoluten Total Retic. Zahl und diesen sog «absoluten» Werten, die eben im Hinblick auf die Schwankungen des BV auch nur relativ sind. In unseren Versuchen war die Übereinstimmung der Total Retic. Zahl mit der relativen (‰) Retic. Zahl sogar besser als mit der Retic. /Raumeinheit (Tabelle III, Abb 5) Im Falle einer Hydrämie kann die Zahl der Retic. pro Raumeinheit niedrig sein, während tatsächlich eine vermehrte Retic. Ausschwemmung vorliegt (s. Zeitpunkt II in Tabelle III und Abb 5)

Bei hämatologischen Erkrankungen mit einer erheblichen Diskrepanz zwischen Erythrozytenzahl/mm³ und absolutem EV

Abschließend sollen die hämatologischen Kriterien, die üblicherweise zur Beurteilung der Erythropoese herangezogen werden, auf ihren Aussagewert bei der Verdünnungsanämie und im allgemeinen geprüft werden (Abb 10)

1 Die Werte der Zellkonzentration (Ery/mm³ Hk) sind auch von der Größe des PV abhängig Sie geben immer dann ein ungenaues Bild der Situation wenn eine Abweichung des BV von der Norm besteht. *Damit muß aber zunächst einmal bei allen schweren chronischen Anämien* (ebenso bei Polyglobulie und Polycythämie) gerechnet werden, weil keiner Anämieform ein bestimmter «Volumentyp» zugeordnet werden kann (5) Bei der Verdünnungsanämie wird diese Diskrepanz zwischen absoluter und relativer Zellzahl ganz besonders deutlich.

2. Die *Bewertung der Retic. Zahl* führt bei jeder stärkeren Regeneration, die mit einer vorzeitigen Ausschwemmung unreifer Retic. einhergeht, zu einer Überschätzung der effektiven Erythropoese, weil die Ausreifungszeit im Blut verlängert ist und die an mehreren Tagen ausgeschwemmten Zellen sich in der Retic. Zahl summieren.

Umgekehrt kann die effektiv Erythropoese auch auf Grund der Retic.-Zahl zu gering eingeschätzt werden, wenn die Ausreifung ganz überwiegend im Knochenmark erfolgt, wie z. B. bei der perniziösen Anämie (24)

Diese Diskrepanz zwischen der Retic. Zahl und dem Ausmaß der Zellproduktion betrifft sowohl die relative wie auch die sog «absolute» und die Total-Retic. Zahl. Auf Grund unserer Untersuchungen möchten wir der relativen Retic. Zahl (‰) den Vorzug geben gegenüber der Retic. Zahl/mm³ weil sie von den Schwankungen des PV unabhängig ist und jeweils den Anteil der Erythrozyten angibt, die sich noch im Retic.-Stadium befinden.

Wenn somit auch die Retic. Zahl zur quantitativen Erfassung der effektiven Erythropoese ungeeignet ist, so bleibt davon ihre große Bedeutung für die praktische Beurteilung hämatologischer Krankheitsbilder unberührt.

3 Das Verhältnis von myeloischen zu erythropoetischen Zellen im Knochenmark (M/E-Index) ist eine relative Größe, die nur bei unveränderter Aktivität der Granulopoese brauchbar ist. Täuschungsmöglichkeiten durch Untermischung von peripherem Blut sind gegeben. Der M/E Index ist ein Maß der *totalen* Erythropoese, die sich bei stärkerer ineffektiver Erythropoese erheblich

von der im peripheren Blut gemessenen *effektiven* Erythropoese (Retic.) unterscheiden kann (22 23)

Für klinische Zwecke ist aber der M/E-Index zusammen mit der Retic. Zahl das brauchbarste Kriterium zur Beurteilung der Aktivität der Erythropoese (17)

4 Das Erythrozytenvolumen, als Resultante von effektiver Produktion und Destruktionsrate, ist ein Maß zur quantitativen Bestimmung der Erythropoese wenn die Lebensdauer der Ery bekannt ist und ein Gleichgewicht zwischen Zellbildung und -abbau besteht.

Im speziellen Fall der Verdünnungsanämie ist auch die Messung der Zellmehrproduktion nach Aktivierung der Erythropoese möglich (6) Die so berechnete mittlere tägliche Produktion ist in Abb. 10 den oben unter 1 bis 3 aufgeführten Größen gegenübergestellt.

5 Zur Problematik der Bewertung des Plasmaeisen turnover und der Inkorporationsrate von Fe^{59} für eine quantitative Erythrokinetik wurde von anderen Autoren Stellung genommen (14 22 57) Wir selbst haben derartige Versuche am Modell der Verdünnungsanämie nicht durchgeführt.

Die meisten der hier mitgeteilten Ergebnisse sind in der Dissertation von D. Spengler (Frankfurt a. M. 1965 In Vorbereitung) enthalten. Dort wird auch auf methodische Einzelheiten naher eingegangen.

Wir danken Frl. M. Atzev für ihre Hilfe bei den Blutuntersuchungen und Frau U Gassow für die Plasmaeiweißbestimmungen.

Zusammenfassung

Durch Infusion großer Mengen eines Plasmaexpanders über mehrere Tage kann bei Kaninchen eine Verdünnungsanämie erzeugt werden, die durch eine Abnahme der Erythrozytenkonzentration (Ery/mm³) bei unverändertem Erythrozytenvolumen charakterisiert ist. Diese Scheinanämie stimuliert wie eine echte Anämie die Erythropoese, es kommt zu einem starken Reticulozytenanstieg und anschließend zu einer Polyglobulie mit Zunahme des Erythrozytenvolumens. Als Folge dieser Polyglobulie tritt eine Hemmung der Erythropoese ein, erkennbar an einer Abnahme der Erythroblasten im Knochenmark und der Reticulozyten im Blut. Die Ausgangswert von Erythrozytenzahl und Blutvolumen werden erst nach vielen Wochen wieder erreicht. Die Lebenszeit der Erythrozyten bleibt während des Versuchs unverändert. Die Bedeutung dieser Befunde im Hinblick auf die Regulation der Erythropoese und die Beziehung von Zellkonzentration und -gesamtmenge wird diskutiert.

Summary

The infusion of large quantity of plasma expander over several days in rabbits produces dilution anaemia characterised by decreased erythrocyte concentration (Ery/mm³) and unchanged erythrocyte olume This pseudo-anaemia, like true anaemia,

stimulates erythropoiesis resulting in a sharp increase in reticulocytes and finally a polyglobulia and increase of erythrocyte volume. The polyglobulia in turn inhibits erythropoiesis which can be recognized by the reduction of erythroblasts in the bone marrow and of reticulocytes in the blood. The original values for erythrocyte count and blood volume are regained only after several weeks. The life span of erythrocytes remains unchanged during the experiment. The implications of these findings for regulation of erythropoiesis and the relationship between concentration and total number of cells are discussed.

Résumé

Par l'infusion pendant plusieurs jours de grandes quantités d'un expanseur plasmatique une anémie par dilution peut être obtenue chez le lapin, anémie caractérisée par une diminution de la concentration des érythrocytes (Ery/mm³) le volume érythrocytaire restant inchangé. Cette soit disant anémie stimule l'érythropoïèse comme une anémie véritable le nombre des réticulocytes augmente fortement puis apparaît une polyglobulie avec augmentation du volume érythrocytaire. Cette polyglobulie est suivie d'une inhibition de l'érythropoïèse qui est reconnaissable à la diminution des érythroblastes dans la moelle osseuse et des réticulocytes dans le sang. Le nombre des érythrocytes et le volume sanguin ne retournent à leur valeur initiale qu'après plusieurs semaines. Le temps de vie des érythrocytes ne changera pas durant toute l'expérience. La signification de ces constatations par rapport à la régulation de l'érythropoïèse et à la relation existant entre la concentration et le volume érythrocytaire sont discutées.

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Bone marrow Sternal bone marrow smears were examined in all patients and their differential marrow composition was established by counting t least 750 cells in each instance. The bone marrow reaction has been classified into (1) normoblastic, (2) macro-normoblastic (3) megaloblastoid and (4) megaloblastic. The normoblastic reaction represented the normal line of erythrocyte maturation while the macronormoblastic reaction showed that at comparable stages of cell development, both the size of the cell as well as of the nucleus was larger than normal. Further the nuclear chromatin was less compact than in normal cells. The megaloblastoid reaction referred to here has also been called typical megaloblastic (12) and 'intermediate megaloblastic erythropoiesis' (7) Compared to the megaloblastic reaction described below the nucleus in megaloblastoid cells was central and the nuclear chromatin coarser The stippled character of the nucleus was maintained so that it presented sieve-like appearance. Pyknosis of the nucleus was seen only in late megaloblastoid cells. In megaloblastic reaction the developing cells were more irregular with eccentric nuclei. The nuclear cytoplasmic ratio was more and the nuclear chromatin very fine and lacy without any evidence of clumping The different lines of cell development described above are shown in Fig. 1

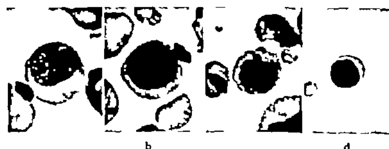


Fig 1 Nucleated red cells at comparable stage of development ( ) intermediate megaloblast, (b) intermediate megaloblastoid cells, ( ) intermediate macronormoblast, (d) intermediate normoblast. × 1500.

Biochemical investigation included estimation of serum bilirubin, cephalin cholesterol flocculation test and determination of serum protein and serum albumin employing standard techniques (9 15)

The *cirrhosis* was classified into 3 categories on the basis of clinical features, mainly of decompensation, biochemical disturbances as shown by decrease in albumin-globulin ratio and positivity of non-specific flocculation tests, and the histological changes seen in the liver biopsy

Animals

Adults albino rats of both sexes of known strain, ranging from 100–150 g in weight were employed to produce cirrhosis. The animals were maintained on standard synthetic diet and allowed water *ad libitum*. For the production of cirrhosis, the animals were *injected carbon tetrachloride subcutaneously twice week, in dosage of 0.1 ml/* 100 g body weight, diluted with an equal amount of liquid paraffin.

Hematologic estimations were made weekly intervals or more often where necessary T study the development of macrocytosis in parallel with cirrhosis, the animals were killed in batches at intervals of 8, 11 and 18 weeks, presumably signifying the period of slight, moderate and marked degree of cirrhosis respectively The animals were sacrificed by exsanguination, the blood subsequently being used for biochemical tests.

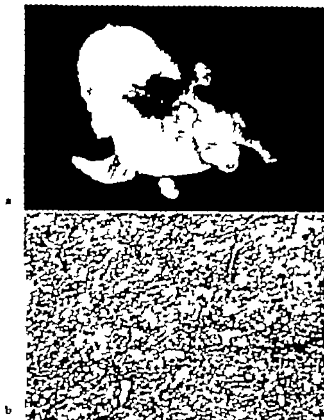


Fig. 2. Gross and microscopic (H + E) appearance of liver in grade I cirrhosis. × 50.

All animals were autopsied. *Grading of cirrhosis* was based on the gross appearances of the liver and its microscopic picture in conformity with the following criteria (17).

Grade I. Grossly the liver showed fine granularity of surface, the granules being less than 1 mm in diameter. Microscopically the cirrhosis was insular or inter-insular. The reticulin fibres were seen as thin strands and the collagen fibres were sparse. There was no attempt at pseudolobule formation (Fig. 2).

Grade II. The granules were slightly coarser, varying from 1–2 mm in diameter. Cirrhosis in these livers was early annular in type. Fibrous septa tended to be thicker and pseudolobule formation had taken place (Fig. 3).

Grade III. The granules were markedly coarse and usually more than 2 mm in diameter. The cirrhosis was annular with marked pseudolobule formation. Reticulin fibres were thick and condensed and collagenisation of fibrous tissue had taken place (Fig. 4).

Besides the changes described above, the liver showed evidence of fatty or hydropic degeneration particularly in animals who died in the early stage. There was variable degree of necrosis and regenerative activity was evident in all instances in the form of hypertrophied liver cells containing one or more macro-nuclei. The vascular changes comprised great reduction in the number of central veins as compared to portal radicals. The portal branches frequently showed dilatation. Bile duct proliferation was often

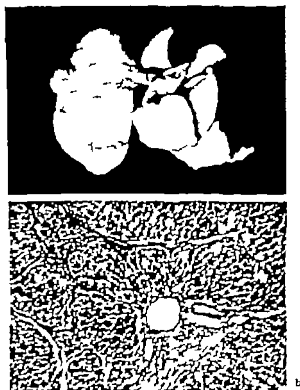


Fig 3. Gross and macroscopic ppearance of liver (Wilder's reticulin stain) in grade II cirrhosis. × 50

seen and the fibrous septa showed infiltration with chronic inflammatory cells, mostly lymphocytes.

I may be mentioned that there was fair degree of correlation between the gross and microscopic appearances, but not so in all instances. I grading cirrhosis, therefore, although gross ppearance of the liver was taken into account, the classification was mainly held on the basis of microscopic picture.

Results

Clinical cirrhosis Measurement of the mean corpuscular diameter in 45 patients of hepatic cirrhosis showed that macrocytosis was present in 25 In these patients the MCD ranged from 8.6 to 11.5 microns with a mean of 9 7 μ (Table I) The MCD of 8 normal subjects averaged 7 6 μ. Of the 45 patients studied, 12 presented with slight (grade I) cirrhosis, it was moderate (grade II) in 10 while 23 patients suffered from severe degree of cirrhosis (grade III) The distribution of MCD in the 3 groups is represented in Fig 5



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Table II
Serum bilirubin and reticulocyte levels in anemic cirrhotic patients

Anemia	No. of patients	Average serum bilirubin mg %	Average reticulocyte %
Normocytic	12	1.8 (0.5–3.0)	1.5 (0.5–3.5)
Macrocytic	25	1.0 (0.5–2.5)	2.5 (0.5–6.0)

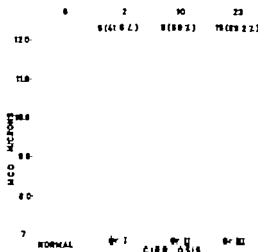


Fig. 5. Variation in red cell diameter (MCD) in relation to severity of cirrhosis.
*These figures represent patients in each group.

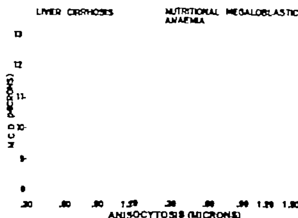


Fig. 6. Comparison of anisocytosis in nutritional megaloblastic anemia and hepatic cirrhosis. Points in the two graphs represent patients at comparable hemoglobin values.

The occurrence of macrocytosis did not bear any relationship either to the severity of anemia, the erythropoietic response as judged by reticulocytosis, or to the level of circulating bilirubin (Table II)

The peripheral blood smears of macrocytic patients showed that the red cells were uniformly enlarged, presenting a picture of what may be called iso-macrocytosis in contrast to the extreme variation in the red cell size met with in other types of macrocytic anemia. As a quantitative measure, the anisocytosis in the patients was expressed in terms of standard deviation of the mean red cell diameter as employed by other workers (3)

Fig 6 represents the degree of anisocytosis in cirrhosis of the liver A group of patients suffering from nutritional megalomegatic anemia was included for comparison Each point in the graph represents a single patient. It is apparent that the variation in the red cell diameter was far more limited in cirrhotic patients in comparison to those of nutritional anemia.

A significant feature was the presence of flattened red cells in 12 of the 25 macrocytic patients, so that a false impression of hypochromia was evident on blood smears. Subsequent determination of the red cell indices showed that in 10 of these 12 patients, although there was an increase in the MCD the mean corpuscular volume (MCV) had remained within the normal limits, thus leading to a decrease in the mean cell thickness (MCT)

The red cell indices of 25 patients showing macrocytosis are summarized in Table I The MVC varied between 80 and 154 μ^3 It was only in 15 patients that the increase in the MCV was accompanied by an increase also of the MCD The mean corpuscular thickness fluctuated widely It was greater than the normal mean in 8 and less in 10 of the patients. In the remaining 7 patients its value was within the normal limits.

Bone marrow Bone marrow smears were examined in all the 25 patients showing macrocytosis. The marrow cellularity was normal or increased in all instances. Two patients showed megaloblastic reaction 15 macro-normoblastic in 3 the bone marrow contained megaloblastoid cells, while in the remaining 5 the picture was normoblastic. In all the patients showing macrocytosis with decreased MCT the bone marrow reaction was macro-normoblastic Differential cellular composition of the bone marrow in these patients is detailed in Table III The significant changes in the bone

Table III
Myelogram in patients with cirrhosis of the liver

Cell type	Normal (8)	Cirrhosis (25)
Blasts	3 0	1.5
Promyelocyte	6.0	3.5
Myelocyte	15.0	16 0
Metamyelocyte	16.0	13.2
Neutrophil	30.0	20.0
Eosinophil	2.0	3.0
Lymphocyte	12.0	5.3
Monocyte	1.0	0.25
Plasma cell	0	1.5
Proerythroblast	1 0	2.5
Normoblasts		
– Basophilic	5 0	13.5
– Polychromic	6 0	12 0
Orthochromic	3.0	6 0
M. E. Ratio	4 8 1	1 7 1

marrow were erythroid hyperplasia, plasmocytosis and lymphopoenia.

Experimental cirrhosis The over all incidence of anemia in 46 rats who developed cirrhosis of the liver was 54 0 %. At the height of anemia the red cell diameters were measured in all the 25 anemic animals. Nine showed macrocytosis. In these animals the mean red cell diameter progressively increased with the severity of hepatic fibrosis. Table IV shows that the normal MCD of 7 40 μ increased to 7.57 7 87 and finally to 8 06 as the liver disease progressed from normal to first, second and third grades of cirrhosis. It further shows that the increase in MCD was not accompanied by a corresponding rise in reticulocytes. Nor was it related to the severity of anemia.

In another group of 10 animals, the mean corpuscular diameters were measured at different periods of carbon terachloride

Table IV
Macrocytosis in relation to the severity of anemia in 25 anemic rats

Group	No. of animals	Average MCD	Hemoglobin %	Retics. %
Normal	80	7 40*	15.2	4 1
Cirrhosis				
Grade I	4	7.57	11.5	4.85
Grade II	6	7.87	9.5	4.73
Grade III	15	8.06	6.8	6.10

*Average of 10 animals only

injection. The results summarized in Table \ clearly reveal a progressive increase in the MCD parallel with hepatic damage The initial MCD of 7.50 microns increased through 7 /6 and 7.96 to 8.04 as the injections were continued for 46 89 and 120 days respectively

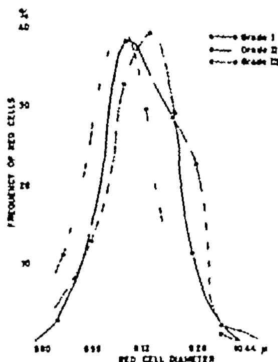


Fig 7 Price Jones curves on animals suffering from cirrhosis of grade I II and III Each graph is derived from the average of animals in the group.

Table I

MCD 1 different periods of CCl injections in rats

Days after CCl4 injection	MCD
0	7 40
46	7 76
89	7.96
120	8 04

Price Jones curves constructed on both the groups of animals conformed to a similar general pattern. The increased MCD was reflected by a shift of the curve to the right (Fig 7) Since variation in the red cell size was minimal the base of the curve was not

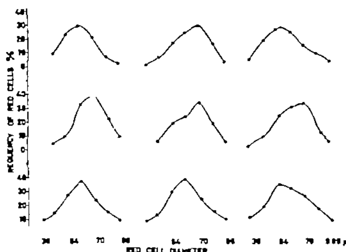


Fig. 8 Red cell frequency distribution curves in 9 cirrhotic rats suffering from macrocytic anaemia.

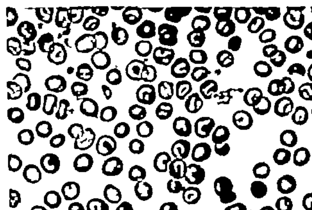


Fig. 9 Flattening of red cells in cirrhotic rat. A number of target cells can be seen

significantly different from the normal. An analysis was made of the red cell frequency distribution curves in all the 9 animals. LARSEN (14) who analysed frequency distribution curves of patients with hepatitis started with the assumption that the curves could be divided into two cell populations, each having a normal distribution around its mean. On this basis he constructed theoretical curves, each curve representing a different proportion between the two cell populations. By finding which of these theoretical

injection. The results summarized in Table V clearly reveal a progressive increase in the MCD parallel with hepatic damage The initial MCD of 7.50 microns increased through 7 76 and 7.96 to 8 04 as the injections were continued for 46 89 and 120 days respectively

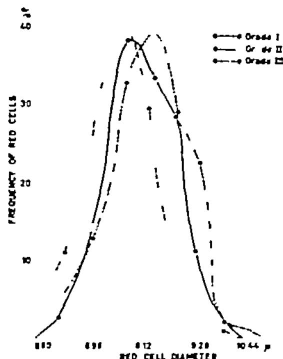


Fig 7 Price Jone curves on animals suffering from cirrhosis of grade I II and III Each graph is derived from the average of animals in the group.

Table V

MCD different periods of CCl injections in rats

Days after CCl_4 injection	MCD
0	7 40
46	7 76
89	7.96
120	8.04

Price Jones curves constructed on both the groups of animals conformed to a similar general pattern The increased MCD was reflected by a shift of the curve to the right (Fig 7) Since variation in the red cell size was minimal, the base of the curve was not

total of 24 injections were given to both the groups. The increase in the mean corpuscular diameter of red cells in the two groups was more or less the same (Table VII)

Discussion

The incidence of macrocytic anemia in cirrhosis of the liver as observed herein was 55.5 / in human subjects and 36.0 / in experimental animals. These figures are in conformity with those reported by other workers. Thus, the frequency of macrocytic anemia in three different series was reported to be 52.8, 64 and 61 / (1 2, 3) In experimental cirrhosis, likewise, macrocytosis was observed in 36 / of the animals, in one study (2) and one third of the animals in another (22)

That the macrocytosis was due to changes in the liver is shown by the increase in red cell diameter in parallel with the extent of liver damage. Further the incidence of macrocytosis was highest in the most severe form of the disease, lesser in grade II and was least in grade I cirrhosis both in clinical as well as experimental material.

The macrocytosis does not appear to be due to the presence in circulation of immature red cells as shown by lack of correlation between the increase in the MCD and the reticulocyte response. Reticulocytes are, on an average, one micron larger than the fully mature erythrocytes (19) It is unlikely that an average increase of 2 1 μ in the MCD of patients with cirrhosis could be produced by reticulocytosis of 2.5 / only

Some workers have considered the osmotic changes in plasma brought by alteration in its concentration of electrolytes, bile acids and proteins due to cirrhosis, as responsible for macrocytosis (18, 21) We did not observe any increase in serum bilirubin or clinical jaundice either in the patients or in animals. Osmotic disturbance as the cause of macrocytosis in liver cirrhosis seems untenable for another reason. It has been shown that the osmotic concentration must be lowered by at least 0 1 / before measurable increase in the red cell diameter occurred (14) The osmotic concentration of 0 1 / sodium chloride solution at 37 C is about 330 mm of Hg Since total concentration of plasma colloids is only 30–40 mm Hg, it is difficult to visualize, how change in this fraction could be the cause of macrocytosis. Further if red cells increased in size

curves best fitted the curve being analysed he determined the diameter of each component In our study only rough determinations of the mean diameter of the various components were made Seven of the 9 curves could be broken down into 2 normally distributed cell populations. The other two showed three components. The curves were skewed in their shape depending on the relative sizes of the components, and whether the smaller component was on the right or the left of the larger (Fig 8) Six curves showed that the bigger component was on the right and that the smaller of the two cell components had a normal MCD

Blood smears revealed little anisocytosis. There was flattening of red cells giving a false appearance of poor hemoglobinization as was observed in human subjects (Fig 9)

The effect of splenectomy on the development of macrocytosis In a group of 13 animals previous splenectomy was performed before the injections of carbon tetrachloride were started. Six unsplenectomized animals were included as control. Both the groups received equal number of injections. The effect of splenectomy was studied in another group of 4 animals. It was observed that the increase in the mean red cell diameter of the splenectomized and unsplenectomized cirrhotic rats was not significantly different. Splenectomy *per se* did not result in any increase in the MCD (Table VI)

Table VI

Effect of splenectomy on macrocytosis of experimental cirrhosis

Animals	Number	Hemoglobin %	MCD
CCl_4 injected	6	9.7	7 73 ± .35
CCl_4 injected and splenectomized	13	12 7	7 68 .40
Splenectomized	4	15.2	7 48 .28

Table VII

Effect of vitamin B_{12} administration on macrocytosis of experimental cirrhosis

Animals	Number	Hemoglobin g	MCD (microns)
Injected with CCl_4	6	11 7	7.8 0.25
Injected with CCl_4 and vitamin B_{12}	9	11.8	7 76 ± 0.30

The effect of vitamin B_{12} administration on macrocytosis A group of 6 rats were given injections of carbon tetrachloride while another 9 received vitamin B_{12} in doses of 25 µg intramuscularly twice a week, in addition to carbon tetrachloride in identical doses. A

tients who suffered from macrocytic anemia, in as many as 20 the bone marrow reaction was abnormal in as much as the nucleated red cells, at the comparable stages of development, where larger than those seen in the normal bone marrow. Two patients showed megaloblastosis, 15 macronormoblasts and in 3 megaloblastoid forms were present in the bone marrow. It is significant that in all the 10 patients who showed thin macrocytosis, the bone marrow reaction was macronormoblastic.

That the macrocytes may be derived from their abnormal precursors in the bone marrow is further suggested by the fact that when the liver damage is prolonged, granulopoiesis is also affected, as reflected by a shift of the myeloid elements to the left, loss of indentation and lobulation of the younger myelocytes and appearance of over segmented neutrophils in peripheral blood (24).

If there is any link between liver damage and the type of bone marrow change, its precise nature is not known. It has been postulated (1) that macrocytosis may be due to macro-normoblastic maturation of cells consequent upon chronic hemolysis of secondary hypersplenism accompanying hepatic disease. Our observation that splenectomized rats subjected to experimental cirrhosis also show similar macrocytosis as the non-splenectomized animals does not support this view. Moreover not all patient of hepatic disease have accompanying hemolytic process. HALL (8) was able to confirm shortened red cell survival with the use of chromium[51] tagged erythrocytes, only in half of the 14 patients with liver disease. He further failed to observe any correlation between the rate of erythropoiesis and the mean red cell diameter. Thus, chronic hemolysis *per se* cannot account for macrocytosis.

It is difficult to visualize that defect in the shortage of vitamin B_{12} contributed to the development of macrocytes. Administration of vitamin B_{12} to cirrhotic rats in adequate doses failed to prevent the formation of macrocytes. There was hardly any difference between the average values of MCD of vitamin B_{12} administered rats and those that did not receive this vitamin. The metabolic functions of liver are so diverse that there is always a possibility of the deficiency of an unidentified factor(s) that may be responsibl for the faulty maturation of red cells. The role of protein in this relation has been suggested (3). Further studies on the role of protein deficiency in affecting the red cell size are required.

Summary

A clinical and experimental study was made of macrocytosis in histologically proven cirrhosis of the liver. Its incidence was found to be 55.5 in human subjects and 36.0% in animals. In both, the increase in red cell diameter paralleled liver damage and the frequency of macrocytic anemia was highest in severe cirrhosis. The macrocytosis did not bear any relation either to the severity of anemia, or the reticulocyte response, or the level of bilirubin in blood. Blood smears showed minimal anisocytosis but there was considerable variation in the thickness of macrocytes. The red cells were thicker than normal in 8 and thinner in 10 of the 25 patients who suffered from macrocytic anemia. I the rest the MCT was within normal limits. Bone marrow changes in patients with cirrhosis indicate that the macrocytes are due to bnormal maturation of their precursors. Neither splenectomy nor the administration of vitamin B_{12} prevented the development of macrocytosis following experimental cirrhosis.

Résumé

Une étude clinique et expérimentale de la macrocytose dans des cas de cirrhose du foie est présentée. Son incidence était de 55,5 chez l'homme et de 36,0% chez l'animal. Chez les deux, l'augmentation du diamètre des érythrocytes était en relation vec le degré de gra ité de l'atteinte hépatique. La macrocytose n avait aucun rapport vec la gravité de l'anémie, la réaction réticulocytaire et le taux de bilirubine du sang. Dans les frottis sanguins, l'anisocytose était minime et les variations de l'épaisseur des macrocytes considérables. Les érythrocytes étaient plus épais que normalement chez 8, et plus minces chez 10 des 25 malades tteints d anémie macrocytaire. Chez les autres malades, l'épaisseur des cellules était normale. Les examens de la moelle osseuse chez les malades, atteints de cirrhose démontrèrent que les macrocytes sont dus à une maturation anormale de leurs précurseurs. Dans la cirrhose expérimentale, ni la splénectomie, ni l'administration de vitamin B_{12} ne peuvent empêcher l'apparition d'une macrocytose

Zusammenfassung

Bei histologisch nachgewiesener Leberzirrhose wurde die Makrozytose klinisch und experimentell untersucht. Ihre Häufigkeit betrug beim Menschen 55,5% und bei Tieren 36,0%. Bei beiden stand die Zunahme des Erythrozytendurchmessers in Beziehung zum Ausmass der Leberschädigung, und bei schwerer Zirrhose war die makrozytäre Anämie am häufigsten. Die Makrozytose zeigte keine Beziehung zur Schwere der Anämie zur Retikulozytenreaktion und zum Bilirubingehalt des Blutes. Im Blutausstrich fand sich eine minimale Anisozytose neben einer beträchtlichen Dickenvariation der Makrozyten. Die Erythrozyten waren dicker als normal bei 8 und dünner bei 10 von 25 Patienten mit makrozytärer Anämie. Bei den übrigen Patienten lag die Zelldicke im Bereich der Norm. Knochenmarksuntersuchungen bei Patienten mit Zirrhose zeigten, daß die Makrozyten *durch* eine anormale *Reifung ihrer Vorstufen bedingt* sind. Bei experimenteller Zirrhose vermochten weder die Splenektomie noch die Zufuhr von Vitamin B_{12} die Entwicklung der Makrozytose zu verhindern.

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Department of Pediatrics of the University Bari (Director: Prof. F. Vecchio) and Naples (Director: Prof. G. Murano)

Electrophoretic Studies of Erythrocyte Glucose-6-Phosphate Dehydrogenase in Normal and Enzyme-Deficient Sardinian Subjects*

F. Vecchio, F. Schettini, L. di Francesco, T. Meloni and G. Rubino

The deficiency of glucose-6-phosphate dehydrogenase (G-6-DPH) of the erythrocytes is characteristic for a group of blood disorders (favism, primaquine haemolytic anaemia, some types of congenital nonspherocytic haemolytic anaemia) (1). The lack of G-6-PDH may be quantitative and also qualitative (2, 3). Favism is a blood disorder of the Caucasian subjects while the primaquine sensitive haemolytic anaemia occurs in Negroes.

The G-6-PDH show electrophoretic differences in these racial groups with a sex linked inheritance. Some phenotypes of G-6-PDH have been identified by electrophoresis of the enzyme from human erythrocytes (4). Three electrophoretic patterns of G-6-PDH were found in erythrocytes of Negroes. A slow G-6-PDH identical in migration to that encountered in Caucasian, a slightly faster G-6-PDH and a broad band representing a mixture of both the slow and fast enzyme (5, 6, 7, 8, 9). These were designated respectively B, A and BA according to the nomenclature of Boyer *et al.* (6). Negro females have an electrophoretic fast, slow or both G-6-PDH; the males have one of the other (9). A fourth electrophoretic variant formerly designated C has also been observed. This variant with very slow electrophoretic mobility has been called 'Baltimore' (6, 10).

In this paper we describe our studies on the electrophoretic mobility of the erythrocytic G-6-PDH in Sardinian subjects carried

* Research supported by a grant from C.N.R., Italy and carried out in the Department of Pediatrics of the University Sassari, Italy (Head: Prof. F. Vecchio).

out with the method of KIRKMAN *et al.* (9) that is of considerable value in population survey (10)

Material and Methods

Ten apparently unrelated mothers and one son from each mother were selected. The children were from 7 days to 13 months old. Furthermore 21 normal subjects, 22 G-6-PDH deficient subjects, 20 G-6-PDH females and 5 premature infants were examined.

The Sardinian origin of the subjects was accurately ascertained.

The experimental procedure was carried out according to the method of KIRKMAN AND HENDRICKSON (9)

Collection of samples: The blood samples of each subject were collected by venipuncture. The heparinized blood was promptly centrifuged at 1000 G for 15 minutes, supernatant plasma and buffy-coat were removed and cold saline solution (0.15 M. NaCl) was added and stirred and the centrifugation was repeated. The supernatant fluid was removed and the packed erythrocytes were added in distillated cold water (1:20) After 10 minutes of standing at 4 °C each haemolysate was transferred into centrifuge tubes and centrifuged at 16000 G for 20 minutes (SERVALL superspeed 85I). The hematocrit with capillary tubes (Cellocrite centrifuge Ljungberg) was determined on packed red cells.

Each stroma-free supernatant was distributed in tubes (0.50 ml in each tube) and stored at —40 °C until the assay

The haemoglobin concentration of stroma-free supernatant was assayed by DRABKIN's method.

Assay of glucose-6-phosphate dehydrogenase was performed by the spectrophotometric method of KIRKMAN AND RILEY (11) and the results expressed in units per ml of haemolysate and per 100 ml of red blood cells. A unit of G-6-PDH was defined as the amount of enzyme necessary for the reduction of 1 μM of TPN per minute at 25 °C.

Preparation of haemolysate for electrophoresis Each supernatant haemolysate was adjusted to 0.05 M. tris/HCl, pH 8.0; 2.7 mM EDTA, pH 7.0; 7 mM β-mercaptoethanol and 0.015 units of G-6-PDH per ml. Haemolysates from G-6-PDH deficient persons were diluted to final concentration of 0.40 g of haemoglobin per 100 ml regardless of activity

If faint turbidity was present in the haemolysate, it was removed by centrifugation t 16000 G per 20 minutes.

Electrophoresis in starch gel: The starch gel was prepared from mixture of 50 ml of 0.5 M. Tris-HCl, pH 8.5; 5.0 ml of 0.27 M. sodium EDTA, pH 7.0; 67 g of hydrolysed starch and 450 ml of deionized water as described by SMITHIES, into 8-Channels vertical electrophoresis tray 5 ml of 1 mM TPN were mixed thoroughly into the molten gel just before it was poured. After few minutes the gel was covered with plastic coperture and placed at room temperature for 2 hours and after at 2 °C for 2 hours.

The slot mould was removed and strip of filter paper was carefully placed in the slots for 20–30 seconds and then removed. Each slot received 50 μl of sample. The slots were covered with molten (50 °C) petroleum jelly The reservoir solution consisted of 0.05 M. Tris-HCl, pH 8 8 0.05 M. sodium chloride and 2.7 mM EDTA-Na pH 7.0. The cathodic reservoir compartment nearest in the gel contained 10 μM of TPN Electrophoresis was performed at 2 °C for 14–16 hours at gradient of 4 volts per cm.

Stain for G-6-PDH The gel was sliced. A mixture of 5.0 ml of 0.5 M. tris-HCl, pH 8.0 0.5 ml of 0.5 M. $MgCl_2$ 3.5 g of starch and 30 0 ml of deionized water were heated until molten and cooled at 45 °C then 1.0 ml of 20 mM G-6-P or 1.0 ml of 20 mM 2-des-

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Department of Pediatrics of the University Bari (Director: Prof. F. Vecchio) and Naples (Director: Prof. G. Mürano)

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Table II

[illegible]rophoretic phenotype of G-6-PDH red cells from sardinian normal and enzyme deficient or intermediate subjects.

Normal			Enzyme deficient				Enzyme intermediate			
Sex	G-6-PDH [illegible]	G-6-PDH phenotype	N.	Sex	G-6-PDH [illegible]	G-6-PDH phenotype	N.	Sex	G-6-PDH [illegible]	G-6-PDH phenotype
M	301	B	1	M	10	B–	1	F	75	B
M	224	B	2	M	0	B–	2	F	36	B
F	194	B	3	M	37	B–	3	F	28	B
M	235	B	4	M	0	B–	4	F	107	B
M	90	B	5	F	0	B–	5	F	104	B
F	147	B	6	F	0	B–	6	F	162	B
M	335	B	7	M	0	B–	7	F	64	B
M	139	B	8	F	0	B–	8	F	118	B
F	291	B	9	M	0	B–	9	F	86	B
M	128	B	10	F	0	B–	10	F	59	B
M	143	B	11	M	12	B–	11	F	101	B
M	198	B	12	M	10	B–	12	F	83	B
M	132	B	13	F	3	B–	13	F	76	B
M	163	B	14	M	0	B–	14	F	85	B
M	212	B	15	F	20	B–	15	F	68	B
M	186	B	16	M	0	B–	16	F	95	B
M	161	B	17	F	0	B–	17	F	53	B
M	258	B	18	M	0	B–	18	F	123	B
M	186	B	19	M	0	B–	19	F	78	B
M	227	B	20	F	19	B–	20	F	150	B
M	220	B	21	F	0	B–				
			22	M	0	B–				

The electrophoretic phenotype with 2d-G-6-P is of the slow type B.

2 females, have the same migration of type B observed in the mothers (Fig 1)

Phenotype of erythrocyte G-6-PDH from normal and enzyme deficient subjects (Table II) The electrophoretic migration in 21 unrelated normal subjects (18 males and 3 females) has been of the slow type B The enzyme in 22 G-6-PDH deficient subjects shows a very little band with slow migration of type B– in 20 intermediate females the enzyme shows a normal slow migration of the type B (Fig 2)

In 7 subjects with G-6-PDH deficiency we have also investigated the pattern of migration of 2-desoxy-G-6-P It has been the same for all tested subjects and has been defined of the slow type B for comparison with a control type B

Phenotype of erythrocyte G-6-PDH from premature newborns (Table III) Five premature infants aged from 4 to 15 days, and of foetal



Fig 1 Patterns of G-6-PD after migration on ertical starch gel electrophoresis. Channels contain th following samples *1* son 5 *2* mother 5 *3* son 6, *4* mother 6, intermediate *5* son 7 *6* mother 7 *7* son 8, *8* mother 8. All G-6-PD types are B.

Fig 2. Patterns of G-6-PD after migration on ertical starch gel electrophoresis. Channels contain the followi g samples *1* female enzyme intermediate n.7 *and 3* negro type A, *4* negro female type B.\ *5* normal n. 6, *6 and 7* negro type A, *8* female enzyme intermediate n. 10. The types of channels 1 5 nd 8 are B.

Table III

Electrophoretic phenotype of G-6-PDH red ells in prema ure infant of fetal ge of seven months.

[illegible]	[illegible]	[illegible]	G-6-PDH [illegible]	G-6-PDH [illegible]
1	M	15	1 92	B-
2	M	7	240	B
3	F	5	58 5	B
4	F	4	341	B
5	F	4	298	B

age of seven months, have been investigated One was a male with enzyme deficiency one was an intermediate female and three were normal The electrophoretic migration of the G-6-PDH in these infants is not different from the migration of the children and adult subjects. The picture has been of the slow type B

The phenotype observed by vertical starch-gel electrophoresis in Sardinian normal subjects European caucasian) has only been the phenotype B that corresponds to the slow migration of the enzyme

A little band of enzyme activity with the same mobility as in normal subjects has been observed in subjects with erythrocyte enzyme deficiency this band is identified as phenotype B

The studies of the inheritance of the gene for G-6-PDH and of the electrophoretic phenotype of the enzyme have demonstrated the existence of two genetic polymorphisms of G-6-PDH. One is characterized by enzyme deficiency the other by an electrophoretic variant. Both are determined by X-linked loci (6 9 10).

In subjects of Caucasian origin the first studies (5–9) have only shown the phenotype B. These researches carried out on unselected subjects of Sardinia confirm the presence of the phenotype B with genetical transmission also in this population.

We have not observed electrophoretic variants as those referred by PORTER *et al.* (10) on the samples of blood from Sardinian subjects of the north-eastern area of the isle. These variants have been defined as Sardinia, Sardinia 2 and Sardinia/Baltimore. Moreover a faster electrophoretic migration of the enzyme than in normal subjects had been observed (3) in U.S.A. in several males, with a low level of erythrocyte G-6-PDH, in a family of italian origin.

The presence of almost two polypeptide chains in the G-6-PDH has been suggested (12) and it has been also indicated that the electrophoretic variants of the enzyme are due probably to the mutation of the aminoacid sequence of the polypeptide chains (10). We have observed the electrophoretic migration of the G-6-PDH in premature infants. But also in these subjects the pattern of the enzyme has always been of the type B. Therefore a foetal type of the enzyme detectable by starch gel electrophoresis, as it exists for the haemoglobin, has not been demonstrated.

The sardinian population is a homogeneous group where the enzyme deficient subjects exhibit several abnormal biochemical characteristics of the G-6-PDH. This enzyme variant has been defined as Mediterranean variant and has an electrophoretic migration of the type B (13). Our findings raises the possibility that the Mediterranean variant of the G-6-PDH with the phenotype B may be a prevalent one among these enzyme deficient subjects.

Acknowledgment. We are grateful to Dr HUGH N KIRKMAN Oklahoma City for the samples of blood of Negroes with G-6-PDH type A and AB.

Summary

Studies of erythrocyte G-6-PDH by means of vertical starch-gel electrophoresis were carried out in sardinian subjects. A slow migration of the enzyme of type B was found in subjects with normal G-6-PDH and in intermediate females. The electrophoretic pattern in G-6-PDH deficient subjects has been of the type B–. The phenotype B is also present in erythrocyte G-6-PDH of premature infants.

Résumé

Des études de la G-6-PDH érythrocytaire à l'aide de l'électrophorèse sur gel d amidon ont été faites chez des personnes sardes. Une migration lente des enzymes du type B se trouv [illegible] hez des personnes [illegible] yant une G-6-PDH normale et chez des femmes i termédiaires. L image électrophorétique est chez les personnes [illegible] yant un manque en G-6-PDH du type B- Le phénotype B se trouv [illegible] ussi dan la G-6-PDH érythrocytaire d enfants prématurés.

Zusammenfassung

Bei Einwohnern [illegible] on Sardinien wurde die G-6-PDH der Erythrozyten mit Hilfe der [illegible] ertikalen Stärkegel-Elektrophorese untersucht. Eine langsame Wanderung des Enzymes [illegible] om Typ B fand sich bei Personen mit normaler G-6-PDH und bei Intermediären Frauen. Bei Mangel [illegible] G-6-PDH zeigte das elektrophoretische Bild den Typus B. Der Phänotyp B kommt auch in der G-6-PDH der Erythrozyten von Frühgeburten [illegible] or

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Hémoglobines anormales chez des malades marocains cancéreux

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Une étude de l'hémoglobine a été pratiquée chez des malades marocains cancéreux, à la suite de la constatation d une Hb A–C dans un néoplasme de la langue. L'intérêt de la recherche d une éventuelle association entre cancer et hémoglobinopathie est renforcée du fait que des hémoglobines anormales C, S K, ainsi que F et A_2 (dans la thalassémie) ont été identifiées chez les marocains. D après les statistiques des laboratoires de Casablanca et Rabat (années 1956–1964) la thalassémie est la plus fréquente parmi les anomalies de l'hémoglobine rencontrées dans la population autochtone qui habite la côte (1,2%) Un nombre restreint de cas a été dépisté pour les autres hémoglobines.

Matériel et méthodes

Notre série comporte 50 malades adultes, hommes et femmes marocains, qui ont pas subi des traitements au préalable. Aucune sélection été faite concernant le stade ou la localisation de la maladie tube digestif, vessie, prostate, utérus et sein.

L'hémoglobine été préparée sur du sang frais dans un délai de 24 h et stockée par la suite à 25 °C jusqu'au moment de son utilisation.

Pour l'identification des hémoglobines A, A_2, F et C, nous avons pratiqué l'électrophorèse sur papier (tampons citrate pH 4,9 et véronal pH 8,6 et 9,2) en gel AGAR (tampon citrate pH 5,2 et véronal pH 8 6) et en gel d'amidon (tampon borate pH 8,6).

Le pourcentage Hb F (R.D.A.) été déterminé d après la méthode de Singer *et al.* (1)

Pour l'Hb A_2 nous avons adopté une méthode visuelle de comparaison des fractions provenant des malades avec un témoin dosé à 3,5%

Les taux supérieurs à 3,5% d'Hb A_2 et 2% d'Hb F ont été considérés comme pathologiques.

Résultats

Sur la série étudiée, 14 malades (28 %) ont présenté des hémoglobines anormales, avec la répartition suivante Hb F (R D A)

dans 7 cas, à des taux variant entre 3 20 et 23 % Hb A_2 dans 8 cas, à un taux supérieur à 3 5 %, Hb C dans 2 cas. Trois des malades portaient à la fois des hémoglobines A_2 et F

Discussion

La thalassémie, maladie connue dans le bassin méditérranéen a été trouvée au Maroc, associée à l Hb S par Mechali *et al* (2) D autres auteurs ont publié la présence d Hb F et A_2 dans des cas de maladies hématologiques comme la leucémie aiguë et chronique (3 4) la maladie de Di Guglielmo (4) le myélome multiple (5) et l anémie aplastique (3)

Chez les malades marocains l'Hb F s est avérée fréquente (7 fois) et en quantité importante (19-23 %) dans les cancers de l estomac. La fraction d hémoglobine A_2 dépistée 8 fois à un taux supérieur à 3 5 % a été trouvée chez des sujets avec une Hb F (RDA) normale ou une Hb F (la RDA inférieure à 2 %) augmentée Trois cas ont eu à la fois Hb F et A_2. Une troisième association a été constatée entre cancer et hémoglobine C chez 2 autres malades.

Ces résultats montrent que les modifications ont portées sur les chaines α et β (6 7) Le pourcentage élevé des sujets marocains ayant à la fois un cancer et une hémoglobine anormale permet de soulever l hypothèse d une relation génétique ou pathologique entre les deux maladies. Nous n avons pas eu la possibilité d étudier l hémoglobine chez les mêmes malades au cours des divers stades d évolution du cancer ni chez les membres de la famille Dans l'avenir un tel travail nous aidera à mieux comprendre l association entre cancers et hémoglobines anormales.

Remerciement Nous remercions le Docteur R. Caraussi (Centre de Transfusion Sanguine et d'Hématologie Toulouse directeur Professeur J R [illegible] pour l'aide technique et les indications qu il a bien voulu nous donner

Résumé

Une étude a été faite sur l'hémoglobine de 30 malades marocains cancéreux 14 cas ont présenté une hémoglobine anormale L'hypothèse d'une relation génétique ou pathologique entre les deux maladies mérite d'être retenue

Summary

Fourteen out of fifty Moroccans with cancer presented an abnormal haemoglobulin. The authors propose the hypothesis of genetic or pathological connection between cancer and the haemoglobulin anomaly

Zusammenfassung

Von 50 marokkanischen Krebspatienten zeigten 14 ein anormales Hämoglobin. Es wird die Hypothese einer genetischen oder pathologischen Beziehung zwischen Krebs und Hämoglobinanomalie aufgestellt.

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Fatal Pulmonary Embolus in Congenital Fibrinopenia

Report of Two Cases

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Fatal pulmonary embolus with pulmonary infarction occurring in a patient suffering from congenital fibrinopenia has previously been reported only once (10). We describe two similar cases of this apparently paradoxical situation.

Case Reports

Case 1 (E. E.) Female

Clinical history: The earlier history and investigations have been described in detail elsewhere (2, 4, 9); intensive studies failed to reveal any haemostatic abnormality other than absence of clottable fibrinogen in the plasma.

In summary, the patient was born in 1932, of parents who were first cousins, and died in 1963. She suffered repeatedly from prolonged post-traumatic bleeding, following cuts, contusions or dental extractions, but bleeding could always be controlled by infusion of fibrinogen. Occasional haemarthroses appear always to have been traumatic, and until her last illness there was no evidence of spontaneous bleeding other than superficial bruises.

In 1954 the spleen was removed following traumatic rupture. This event probably was of great significance in playing a part in her final illness.

Menstruation started at 15 years, and despite fears of uncontrollable loss proved entirely normal up to the time of her marriage at the age of 26 years. Thereafter there were episodes of menorrhagia which led to recurrent iron deficiency anaemia, and an endometrial curettage was performed in May 1962, under fibrinogen cover.

In November she was readmitted with a haemoglobin of 37% (5.4 g%) on account of further menorrhagia and total hysterectomy was performed on November 15th 1962, covered by fibrinogen infusions, and without undue bleeding until 20th November (see below). Further haemorrhage from the vaginal vault occurred on December 4th and 18th requiring packing, but for the next two months she remained well.

In early March 1963 admission became necessary for severe left sciatic pain present for five weeks. There appeared to be no precipitating factor and the pain was constant. Four days after admission she had a sudden severe pain in the right buttock which was found to be due to a large haematoma. This occurred spontaneously while the patient was lying in bed. The bleeding was considerable, the haemoglobin dropping from 67% (9.8 g%) to 46% (6.7 g%). As a result of numerous intravenous infusions over the years, the superficial veins had become so disorganised that intravenous in-

fusions were now only possible via a polyethylene catheter introduced into femoral vein, through which blood and fibrinogen were given over the next eight days. Unfortunately the catheter was allowed to remain in the same vein throughout this time. Two days after the start of the infusion, the patient complained of right-sided pleural pain, cough and blood-stained sputum. A blood-stained pleural effusion developed. Culture of the sputum and pleural fluid were sterile. An E.C.G. showed S_1-Q_3-T pattern. A pulmonary embolus was diagnosed; the episode settled and she was discharged home on April 7th 1963.

Apparently she became ill within few days, with vomiting, fever and coughing up blood. She spent most of the time in bed but did not at first seek advice, although when she attended Outpatients on April 18th it was clear that she was very ill. Her weight had fallen by one stone (ca. 6 kg) she was febrile, her temperature was 38.3 °C and the right pleural effusion had re-accumulated. The right femoral vein was palpable as hard cord. A new feature was extensive bruising on the trunk and limbs, the bruises having pale centres. A blood sample was obtained by arterial puncture, which gave rise to an enormous haematoma. Blood cultures remained sterile but the white cell count was 24,000 mm^3 and within few days the platelet count began to rise, reaching 1,027,000 mm^3 by May 1st. Despite antibiotics the deterioration continued, spontaneous bruising increased, the dyspnoea became worse and the patient eventually died in heart failure on May 7th 1963.

Post-mortem findings: Postmortem examination showed rather thin, young, edentulous woman with abdominal splenectomy and hysterectomy scars and an old scar situated below the left patella. The main internal findings included total collapse of the right lung by purulent pleural effusion amounting to about one litre. The lower lobe of the right lung was largely replaced by necrotic infarcted tissue and the main artery supplying this lobe was filled with pale thrombotic embolus. The right common iliac vein contained pale crumbling antemortem thrombus from which the embolus probably arose. The left lung was oedematous and the left pleural sac was partly obliterated by old adhesions. There were small petechial haemorrhages in the mucosa of the stomach and haematomas were present on the right side of the urinary bladder and in the perarticular tissues of the left shoulder joint. The liver was pale and fatty. The major arteries showed no atheroma.

Microscopically the principal changes included necrotic pulmonary infarct with overlying acute pleurisy in the lower lobe of the right lung caused by eosinophilic platelet thrombi obstructing the adjacent branches of the pulmonary artery. Organising platelet thrombi were present in the right common iliac vein but none of these thrombi contained stainable fibrin, although the stains used included Weigert's fibrin stain, phosphotungstic acid stain and the Martius-Scarlet-Blue method (M.S.B.) Masson 44/41 and the Fuchsin-Miller fibrin stain (7). Several lymph glands were sectioned and in one the sinus-lining cells contained unidentifiable eosinophilic droplets surrounding the nuclei. No microorganisms were found in any of the tissues examined.

Death was attributed to cardiac failure secondary to multiple pulmonary emboli causing multiple lung infarcts.

Fate of infused fibrinogen. Previous observations (5) of the fate of infused fibrinogen in this patient prepared from time-expired blood had yielded rates of disappearance of 14 and 15% /day after equilibration had been reached. A further estimate of 16% /day was obtained in 1962 at the time of the dental clearance. On those occasions sufficient superficial veins had still been available to obtain serial blood samples by venepuncture, and the rates of disappearance could be determined from daily observations of plasma fibrinogen concentration. Latterly however it was necessary to obtain almost all blood samples by arterial puncture, and their number was therefore severely limited. Following hysterectomy in November 1962 the monitoring of plasma fibrinogen concentration

was for this reason much less thorough than would have been desirable. In the immediate post-operative period, for instance, no determinations were made between the 15th and the 22nd November during which time the concentration was found to have fallen from 0.31 g/100 ml to bout 0.03 g/100 ml, and by interpolation it appeared that the level would have been about 0.07 g/100 ml on 20th November 1962 when post-operative bleeding began. Four further infusions of fibrinogen were given over the next two weeks, and the plasma level probably did not fall below 0.10 g/100 ml during this period. Rates of disappearance, based in each case on only two or three determinations of plasma concentration following each infusion, lay between 18 and 22%/day; whereas the initial post-operative value was 26%/day One further estimate of 20%/day was obtained in March, 1963, during the treatment of the haematoma in the right buttock. These values are higher than those previously obtained from this patient, but it must be remembered that the later estimates were based on very few points and therefore represent mean rates over the whole periods of the observations including any initial rapid fall. On this basis, values of up to about 20% probably do not greatly differ from earlier results. In any event there is no evidence of a progressive increase in the rate of removal of infused fibrinogen over the last few months of the patient's life.

Case 2 (N N.), Male

Clinical history A Greek aged 28 who, in 1937 at the age of 24 had been investigated for abnormal bleeding following tooth extractions at University College Hospital and was found to be grossly fibrinopenic.

His final illness began suddenly in mid-February 1961 with fever right-sided pleural pain, cough and haemoptysis. He was treated with Tetracycline but failed to improve and when a pleural effusion developed he was admitted to hospital on 6th March, 1961

Examination showed an ill man. He was sweating, with pallor due to peripheral constriction, and there was a large right pleural effusion. Pulse 120/min, B. P 140/100. The history of abnormal bleeding was btained at this stage and fibrinopenia was confirmed.

In view of the risk of precipitating bleeding, a fibrinogen infusion was given before aspirating the pleural cavity Two litres of heavily blood-stained fluid were removed. It was necessary to give repeated infusions of blood, fibrinogen and plasma in an effort to stop bleeding into the pleura, but in the course of the next fourteen days about eleven litres of heavily bloodstained fluid were aspirated from the right pleural cavity Each aspiration was necessary to relieve severe shortness of breath and falling blood pressure.

An E. C. G. taken on admission showed changes suggestive of posterior infarct. After the original fibrinogen infusion it was noticed that both calves were tender and later one cephalic vein became thrombosed. On the 27th March there was a fairly obvious deep vein thrombosis in the right calf. A bronchoscopy revealed no specific abnormality A thoracotomy disclosed a mass in the right lower lobe which on biopsy was found to be only young fibrous tissue.

Following this operation a tension pneumothorax developed and further operation was undertaken in which the pleura was stripped and the leak from the lung was oversewn. Within a matter of hours, he developed increasingly severe failure and died.

Post-mortem findings: Postmortem examination disclosed the body of slightly jaundiced, obese young man with an unhealed incision over the sixth right intercostal space and stab drainage wound in the intercostal space below

The principal changes were present in the cardiovascular and respiratory systems. There was a recent infarct, the centre of which was liquefied, involving the outer part of the lower half of the posterior wall and apex of the left ventricle Despite careful search no obstruction was found in either coronary artery and there was an absence of atheroma

in the major arteries. Both common femoral veins and the deep calf veins in both legs contained pale antemortem thrombus. The lungs showed multiple infarcts in both lower lobes and in the right middle lobe. Some of these infarcts were necrotic, others were in process of organisation, while the remainder of both lungs were extremely oedematous. An infarct in the right middle lobe had ruptured through the pleural surface causing bronchopleural fistula. The left pleural sac contained 300 ml of straw-coloured effusion but the right sac had remained empty since the operation. The liver was pale and fatty and no biliary obstruction was found.

Microscopical examination showed recent and pale infarcts in the lungs one of which was infected with an *Aspergillus sp.* Adjacent branches of the pulmonary arteries contained platelet thrombi in varying stages of organisation but these lacked fibrin (The same stains were used as in Case 1). There was ischaemic fibrosis in the wall of the left ventricle. Death had resulted from myocardial infarction of uncertain aetiology and multiple emboli causing infarcts in the lungs some of which were infected with *Aspergillus sp.*

Plasma fibrinogen concentration This patient does not appear to have been intensively investigated when in normal health, and detailed information on his haemostatic mechanism is not available, but congenital fibrinopenia seems to be the most likely explanation of the results obtained. The findings in 1957 (for which we are indebted to Dr J O. HAWKSLEY) were, whole blood unclotted after 6 h, prothrombin time 31 7 (control 12.2) clot formed by adding thrombin solution (Fibrindex) indicated very low' plasma fibrinogen concentration, electrophoretic fibrinogen band 'just detectable with normal bleeding time (2.5 min), and platelet count (380,000 mm³). On admission to St. Thomas Hospital in 1961, the plasma fibrinogen concentration was less than 10 mg/100 ml by clot-weight method and the prothrombin time was over 2 min. The thromboplastin generation screening test (5) gave normal result and the platelet count was 175,000 mm³ The rate of disappearance of infused fibrinogen was not measured, but the plasma concentrations which were recorded following the infusions did not suggest an abnormal rate of disappearance.

Discussion

Postmortem findings Fibrinopenia is a rare disease and the two cases presented above both provided an opportunity to study the structure of thrombi in the absence of fibrin formation. In both cases the patients died from the effects of thrombotic embolism despite their inability to form fibrin. Normally the majority of pulmonary emboli are mixed thrombi originating in distal leg veins which are the seat of phlebothrombosis. In both of the present cases no fibrin was demonstrable either in the primary thrombi or resultant emboli all of which consisted only of fused platelet masses with some entrapped circulating blood cells.

The changes found in both of our cases closely resembled those described by DE VRIES *et al.* (10) In all three cases the extensive pulmonary infarcts were characterised by liquefaction and by an absence of any fibrin but otherwise resembled normal infarcts in their microscopical features. The liquefactive changes

present in the pulmonary infarcts in both of our cases are unusual in the absence of chronic heart failure and remain unexplained. The possibility that absence of fibrin may have contributed to this change cannot be excluded. The staining properties of fibrin vary according to its age, fresh fibrin being stained by acid dyes of low molecular weight and small size while older fibrin approximates more closely to collagen in its staining properties. For this reason a variety of staining methods should be employed to detect fibrin at all ages. In our cases the failure of five different methods to detect fibrin in either the primary venous thrombi of resultant emboli and also in the lung tissue at the edge of the infarcted areas shows that it was absent both in older lesions and in those formed shortly before death.

With the electron microscope, JOHNSON *et al.* (6, 8) have shown in rat and guinea pig mesentery that haemostatic platelet aggregates formed *in vivo* come to contain fibrin strands among the platelets within a few minutes of the arrest of bleeding. They also demonstrated a fibrin network entrapping red cells. Our results suggest that fibrin formation (at least to the extent identifiable with the light microscope) is not essential to the continuing solidarity of a platelet thrombus.

The fate of infused fibrinogen. In the cases of BRÖNNIMANN (1) and of DE VRIES *et al.* (10) an anti-fibrinogen was demonstrated in the patient's blood before death, destroying infused fibrinogen and rendering both patients refractory to treatment. In our patients, infused fibrinogen appeared (and in case 1 remained) in the circulation as would have been expected, providing good evidence against the development of an anti-fibrinogen in either.

Thrombocytosis in case 1. In case 1 the platelet count rose to over 10^6/mm^3 and many spontaneous bruises appeared, during the terminal illness. We think that this was an example of an exaggerated haemopoietic response such as may be seen in previously splenectomised subjects, and was presumably related to the pulmonary infarct. Work on other patients has shown (MCCLURE, INGRAM, STACEY, GLASS AND MATCHETT in preparation) that when a temporary rise in platelet count occurs even a long time after splenectomy the circulating platelets share some of the abnormalities shown by thrombocythaemic platelets. We therefore suppose that, terminally the platelets in case 1 became haemo-

statically ineffective as the platelet count rose and that this caused the extensive spontaneous bruising which had not hitherto been a feature of her haemorrhagic history

Acknowledgement: We are grateful to Dr. B. CRAMER and to Dr. H. K. GOADBY for their permission to publish these cases.

Summary

The terminal illnesses of two cases of congenital fibrinopenia are described. Both patients died of pulmonary embolism, and postmortem, the lungs of both showed liquefaction in the infarcted areas. A variety of staining techniques failed to demonstrate fibrin either in the original thrombus or embolus or around the infarct. The behaviour of infused fibrinogen showed that neither patient destroyed this material unusually rapidly indicating that neither had developed an antifibrinogen antibody. In one patient the white cell and platelet counts rose to high levels, and extensive bruising appeared; this myeloproliferative reaction was thought to be related to her previous splenectomy

Résumé

Rapport sur le tableau clinique terminal de deux cas de fibrinopénie congénitale. Les deux malades moururent d'embolies pulmonaires. A l'autopsie, les poumons présentèrent une liquéfaction des parties infarcisées. Il ne fut pas possible de mettre en évidence de la fibrine à l'aide de différentes méthodes de coloration, ni dans le thrombus primitif, ni dans l'embolus, ni dans la zone de l'infarctus. Une infusion de fibrinogène démontra que chez ces malades, celle-ci n'est pas dégradée plus rapidement que d'habitude. Il en ressort qu'il ne se trouvait aucun anticorps contre le fibrinogène. Chez l'un des malades, le nombre des leucocytes et des thrombocytes monta très haut, et il se forma des suffusions très étendues. Cette réaction myéloproliférative fut mise en relation avec la splénectomie exécutée antérieurement.

Zusammenfassung

Es wird über das terminale Krankheitsbild zweier Fälle von kongenitaler Fibrinogenopenie berichtet. Beide Patienten starben an Lungenembolie, und bei der Autopsie zeigten die Lungen eine Verflüssigung der infarcierten Bezirke. Weder im ursprünglichen Thrombus, noch im Embolus, noch im Bereich des Infarktes liess sich mit verschiedenen Färbemethoden Fibrin nachweisen. Bei keinem der Patienten wurde infundiertes Fibrinogen ungewöhnlich rasch zerstört, woraus hervorgeht, dass keine Antikörper gegen Fibrinogen gebildet wurden. Bei einem Patienten stiegen die Leukozyten und Thrombozyten auf hohe Werte an, und es bildeten sich ausgedehnte Blutungen. Diese myeloproliferative Reaktion wurde zu der früher vorgenommenen Splenektomie in Beziehung gebracht.

References

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present in the pulmonary infarcts in both of our cases are unusual in the absence of chronic heart failure and remain unexplained. The possibility that absence of fibrin may have contributed to this change cannot be excluded. The staining properties of fibrin vary according to its age, fresh fibrin being stained by acid dyes of low molecular weight and small size while older fibrin approximates more closely to collagen in its staining properties. For this reason a variety of staining methods should be employed to detect fibrin at all ages. In our cases the failure of five different methods to detect fibrin in either the primary venous thrombi or resultant emboli and also in the lung tissue at the edge of the infarcted areas shows that it was absent both in older lesions and in those formed shortly before death.

With the electron microscope, JOHNSON *et al.* (6 8) have shown in rat and guinea pig mesentery that haemostatic platelet aggregates formed *in vivo* come to contain fibrin strands among the platelets within a few minutes of the arrest of bleeding. They also demonstrated a fibrin network entrapping red cells. Our results suggest that fibrin formation (at least to the extent identifiable with the light microscope) is not essential to the continuing solidarity of a platelet thrombus.

The fate of infused fibrinogen. In the cases of BRÖNNIMANN (1) and of DE VRIES *et al.* (10) an anti fibrinogen was demonstrated in the patient's blood before death, destroying infused fibrinogen and rendering both patients refractory to treatment. In our patients, infused fibrinogen appeared (and in case 1 remained) in the circulation as would have been expected, providing good evidence against the development of an anti-fibrinogen in either

Thrombocytosis in case 1. In case 1 the platelet count rose to over $10^6/mm^3$ and many spontaneous bruises appeared, during the terminal illness. We think that this was an example of an exaggerated haemopoietic response such as may be seen in previously splenectomised subjects, and was presumably related to the pulmonary infarct. Work on other patients has shown (MCCLURE, INGRAM, STACEY, GLASS AND MATCHETT in preparation) that when a temporary rise in platelet count occurs even a long time after splenectomy the circulating platelets share some of the abnormalities shown by thrombocythaemic platelets. We therefore suppose that, terminally the platelets in case 1 became haemo-

World Federation of Haemophilia

The Third Congress of the World Federation of Haemophilia was held in Paris, September 7th to 9th 1965, sponsored by The International Society of Blood Transfusion. The Federation was founded in Copenhagen in 1963 following suggestion by Dr E. NEUMARK (London) and Dr J P SOULIER (Paris) echoed by Sir WELDON DALRYMPLE-CHAMPNEYS in presidential address to the Haemophilia Society and stimulated by Mr F SCHNABEL (Montreal) president of the Canadian Haemophilia Society The second congress was held in Amsterdam in 1964 under the presidency of Prof. S. VAN CREVELD.

In the session on the detection of *carriers of haemophilia* with Prof. K. M. BRINKHOUS (USA) in the chair lively discussion developed, but everyone agreed that possible carrier could not be identified with certainty by laboratory tests at present and nobody was prepared to say whether woman was or was not carrier in haemophilic family C. KERR (Australia) thought that haemophilia is transmitted under polygenic control. M. VERSTRAETE (Belgium) used capillary blood but his results were equally indefinite.

In session on the *treatment of pain in haemophilia* the continued widespread use of aspirin and salicylates was deplored by several speakers. P WOLF (London) P IZARN (France) and B. NOEL (France) stressed the usually prompt alleviation of pain in joint nd muscle haemorrhage following an infusion of plasma and pleaded for restraint in the f analgesics, early immobilisation when a limb was involved, but also early restora- f movement in joint.

rious papers were given by teams from Sweden, Switzerland, Russia, Israel on surgery in haemophilia, the necessity f vaccinating haemophilic pa- treatment of haematuria, which is frequent complication in haemo- avated by salicylates.

advisers of the national haemophilia associations reported progress (Argentine) described the laboratory control and production of that haemophilia patients should not remain longer than ab- tal, but should return as soon as possible to their own sur la) told of society in New South Wales and of another oped on different lines, but had friendly relations. A. as medical and scientific society on haemophilia in which looked after the patients interests. C. HARRIS of medical and social matters caused an uneven patients were looked after satisfactorily J P and blood products of the more than 1000 organisations and their relationships with York) showed the special identity *Foundation and discussed some of* oese caused by haemophilia. E. ppreciated that familial cases of ; the others could be sporadic or ANDALAKI (Greece) R. DELLA R. BAKLAJA (Yugoslavia) re DE NICOLA (Italy) and O the care of haemophilic pa-

The *haemophilic at school* presents special problems. D. Alagille (France) described the medical and academic supervision in the two boarding schools and the recently gained permission of haemophilic boys to attend girls schools. K. Dormandy (London) told of the reply to her questionnaire sent to 150 families with haemophilic children in South-Eastern England and of the plan to set up a boarding school for them near Oxford. The *psychological disturbances* in the haemophilic school boy whether at home, at school or board were stressed in several papers.

The *working life of the haemophilic* was the subject of a session under the chairmanship of H. Chaigneau (President of the French Haemophilia Society who was a perfect host throughout the meeting) P. Izarn (France) suggested that working as a clerk or accountant for those who were sufficiently well educated, and as an electronic mechanic was the best solution and that work with sharp tools was contra-indicated. E. Neumark (London) said that rehabilitation must start as soon as an acute episode was overcome. Prolonged stay in hospital can lead to gaps in education which are difficult to close later on. Illness may lead to the loss of a job and sometimes to the loss of the discipline of life and of work. Much effort is needed to avoid a man becoming unemployable.

Delegates did not think that an international identity card for haemophilic people was necessary. The World Federation of Haemophilia has become an Associated Member of the International Society for Rehabilitation of the Disabled. Its next meeting will be in Montreal in 1967. E. Neumark, London

Varia

Standardisierung in der Hämatologie

Vom 14. bis 18. Juni 1965 wurde ein erstes internationales Standardisierungs-Symposium für die Hämatologie in Südamerika abgehalten. Die Tagung wurde in Ica (Peru) unter dem Vorsitz von Dr. E. Rewald (Argentinien) abgehalten; 2. Vorsitzender war R. Eilers (USA). Die Kurzfassungen der Vorträge sind von der Peruanischen Hämatologischen Gesellschaft in einem Heft herausgegeben worden. An der Sitzung wurde die Notwendigkeit der Standardisierung hämatologischer Methoden betont und die Gründung nationaler Standardisierungskomitees in den einzelnen amerikanischen Ländern angeregt. Am 11. Dezember 1965 fand in Mar del Plata eine zweite Sitzung statt.

Acta haemat. 35: 65–79 (1966)

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The Central Nervous System in Regulation of Erythropoiesis

S. Halvorsen

The concept of central nervous system (CNS) participation in the regulation of erythropoiesis was originally based on clinical and experimental investigations showing a correlation between CNS disorders and polyglobulia, and polyglobulia following stimulatory or ablative procedures on the diencephalon. Most of this work is now considered inconclusive because only peripheral blood values were investigated. A recent clinical study has, however, provided well documented evidence in favor of an influence on erythropoiesis from the CNS (11). The CNS participation in erythropoiesis regulation has on the other hand by many workers been considered as non-existent or non-specific. The case reports of Stohlman *et al.* (38) and Schmid and Gilbertsen (34) have been considered to exclude the organs above the diaphragm as of primary importance for the regulation of erythropoiesis. Thus there is at present considerable controversy regarding the role of the CNS in the control of erythropoiesis. The purpose of the present paper is to summarize and analyze our own studies and to review other recent studies in an attempt to evaluate the data regarding this problem, and to draw some conclusions for future studies. Beer (4), Seip (35), Komiya (23), Hollan (20), Reissmann (33) and Baciu (2) have reviewed the extensive literature on this problem and these reviews should be consulted for a more detailed description of the previous studies.

The working hypothesis for our studies on the CNS control of erythropoiesis has been that the variations in oxygen tension are recorded in the hypothalamic area, and that the erythropoietin(s) are produced in one or several target organs stimulated via hypo-

thalamus by humoral or nervous routes. Our studies have therefore concentrated on the influence of hypothalamus on erythropoiesis. They have been divided into three types of experiments

(1) Stimulation experiments Electrical stimulation of the hypothalamus using reticulocyte counts, red cell mass and plasma erythropoietin levels as parameters (36 13)

(2) ablation experiments Hypothalamic lesioning procedures followed by short term hypoxia using reticulocyte counts as parameter The hypoxic tests were performed with and without pretreatment with pituitary hormones (16)

(3) hormone experiments Comparison of the effect of pituitary and target organ hormone administration with the effect of electrical stimulation of the hypothalamus (14 17 18)

The studies have been published in detail elsewhere and only the data used in the comparison between the effect of hypothalamic stimulation and pituitary hormone administration are included in this report. The data from the ablation experiments are not reported but are referred to in the discussion.

Material and Methods

The experiments were performed in adult male rabbits, except in the stimulation studies where both male and female rabbits were used. The reticulocyt counts and the red cell mass per kg body weight (RCM/kg) differed slightly in the various groups, but since relative more than absolute values are compared, this has not been considered significant. The experimental period has been 14 days, in some of the hormone experiments an additional 14 days. The red cell mass (RCM) was determined with the Cr^{51} method as previously described (14 36). The reticulocytes were stained with the brilliant cresyl blue method (35) and 5000 or 2000 erythrocytes were counted. The plasma erythropoietin was tested in starved mice and intact mice (13) and in polycythemic mice (13, 47).

Results

Following electrical stimulation of the hypothalamus 8 of 28 rabbits showed a marked rise in reticulocytes and red cell mass (36) These 8 rabbits are referred to as the positive response group, and data from this group only are included here. Ten rabbits showed no reticulocytosis and no increase in RCM while 10 rabbits showed inconsistent variations in these parameters. These groups are referred to as the negative and the doubtful response groups, respectively As the positive response group was a selected group, it has not been found justified to perform statistical evaluation of the differences between the groups.

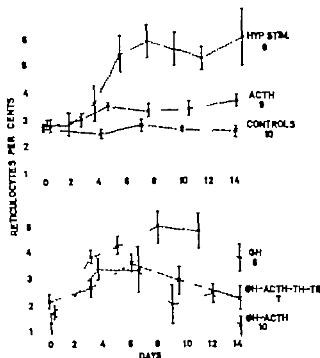


Fig 1 Upper figure Mean reticulocyte response to hypothalamic stimulation (positive response group) and to ACTH injections in intact rabbits compared with the reticulocyte values in control rabbits.

Lower figure Mean reticulocyte response to porcine growth hormone (GH), GH-ACTH, and GH ACTH-thyroxin-testosterone (GH-ACTH-TH-T) The brackets indicate 2 standard errors of the mean. The figure below the group name indicate the number of rabbits in each group.

Fig 1 shows the mean *reticulocyte* response in the various types of experiments. The intact control rabbits had very stable reticulocyte counts indicating that this method is also useful in rabbits as a measure of erythropoiesis. The rabbits in the positive response group showed a marked rise in reticulocytes from the second or third day following stimulation, and the response differed markedly from the controls. ACTH increased the reticulocytes slowly but the increase was significantly different from the controls and also different from the positive response group. Both growth hormone (GH) alone and GH in combination with ACTH (AC) increased the reticulocyte counts and the responses could not be differentiated from the response to hypothalamic stimulation. GH-

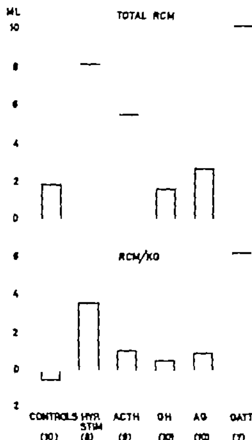


Fig 2. Upper figure Mean of the differences between red cell mass (RCM) determinations after a 2 week experimental period and the values before this period in intact, control rabbits, in the positive response group of hypothalamic stimulation and in rabbits injected with ACTH, GH, ACTH-GH (AG) and with GH-ACTH-thyroxin-testosterone (GATT)
Lower figure Mean of the differences in RCM-kg body weight in the same groups. The number of rabbits in each group is given below

ACTH thyroxin-testosterone (GATT) also increased the reticulocytes, but less than GH alone and GH ACTH in combination.

Fig 2 and Table I show the mean changes in *total RCM* and *RCM/kg body weight* in the various experiments. The results are expressed as the mean of the differences between the values after a 14 days experimental period and the values before this period. The controls showed a slight, insignificant rise in total RCM and a slight decrease in RCM/kg body weight. ACTH increased total

RCM and with a slight increase in RCM/kg body weight. In the GH treated group the total RCM increased less than in the controls, while the RCM/kg body weight increased relatively more due to weight loss of the animals. GH combined with ACTH increased the total RCM less than ACTH alone, while the RCM/kg body weight increased slightly GH ACTH thyroxin-testosterone increased the total RCM markedly and more than in any of the other groups including the positive response group of hypothalamic stimulation. The RCM/kg body weight also increased markedly but this rise may give a false impression of the magnitude of the response because of weight loss of the animals. Electrical stimulation of the hypothalamus increased the total RCM and RCM/kg body weight more than did ACTH, GH and GH ACTH administration, but less than GH ACTH thyroxin testosterone.

Table I also lists the mean changes in the other parameters studied. *Hemoglobin, hematocrit* and *number of erythrocytes* did not change in the control group or in the ACTH injected group. In the positive response group the hematocrit increased moderately In the GH and GH ACTH injected groups, the peripheral blood values dropped initially but at the end of the two week period the levels were only slightly below the initial values. In the group of rabbits injected with GH ACTH-thyroxin testosterone, the hemoglobin, hematocrit and number of erythrocytes increased to levels significantly above the other groups. The mean corpuscular volume (MCV) increased in the positive response group less in the GH, ACTH and GH ACTH injected groups while there was no change in the other groups.

Table II summarizes the results of the *plasma erythropoietin* determinations. Plasma from rabbits subjected to electrical stimulation of the hypothalamus for 2 weeks increased the reticulocytes and the Fe^{59} erythrocyte uptake more than plasma from control rabbits when tested in starved mice. Plasma from one of these rabbits also increased the reticulocytes and the Fe^{59} erythrocyte uptake in polycythemic mice. Plasma from the hormone injected rabbits withdrawn after 2 weeks of administration did not increase the Fe^{59} erythrocyte uptake significantly neither in starved nor in polycythemic mice. Plasma withdrawn from the rabbits injected with GH ACTH-thyroxin testosterone after the development of polycythemia seemed to inhibit erythropoiesis in starved mice.

Table I

differences between the values after and before an experimental period of 2 weeks (± s. e [illegible] ts receiving electrical stimulation of the hypothalamus and rabbits injected with ACTH, GH and GH-ACTH-thyroxin-testerone

	Control	Hypothalamic stimulation Pos. response	ACTH	GH	GH-ACTH
	1.8±.2	9.1±1.5	3.5±1.5	1.6±1.3	2.7±1.7
l	−0.4±0.5	3.1±0.3	1.1±0.3	0.5±1.0	0.9±0.8
g	0.0±0.3	−0.3±0.3	−0.2±0.3	−0.3±0.4	−0.6±0.4
, mill.	0.[illegible]±0.3	−0.1±0.3	−0.2±0.2	−0.4±0.2	−0.4±0.1
	−0.9±1.1	3.0±0.6	0.9±0.5	−0.3±0.9	−1.2±0.9
	−4.6±4.5	7.9±3.6	3.6±1.8	4.0±2.9	3.6±2.6

Table II

[illegible]ropoietin levels in rabbits following [illegible] weeks of hypothalamic stimulation (positive response [illegible] by injections of ACTH, GH, GH-ACTH and GH-ACTH-thyroxin-testosterone for 2 week[illegible] see 13 14 17 18)

[illegible]	Intact mice	Starved mice		Polycythem[illegible]	
[illegible]	Reticulocytes	Reticulocytes	Fe^{59} uptake	Reticulocytes	
[illegible]nse group	increased	increased	increased	increased	i
	insignificant increase		insignificant increase		
					i
					i
					i
					i
Thyroxin-testosterone			inhibition (?)		i
					i

Discussion

The concept of a humoral regulation of erythropoiesis through erythropoietin is generally accepted but the details in the mechanism are unknown for several steps. Hypoxia is considered to be the fundamental stimulus for erythropoietin production but there are few data regarding the site of recording of the variations in oxygen tension. Although perfusion studies of the kidneys (8, 24) and experiments with clamping of the renal artery (19) suggest that both the registration of oxygen tension and the production of

erythropoietin may take place in this organ, neither of these functions can be located in the kidneys exclusively because erythropoiesis continues in the anephric man (38) and animal (28). The site of action of erythropoietin is still unclear and the reticulocyte release mechanism almost unknown. The effect of the hormones is no longer considered to be so definitely non-specific since recent work on thyroxin (27), testosterone (10), GH (9, 33) and prolactin (9, 21) provide evidence that these hormones may act directly in the bone marrow or stimulate erythropoietin production. These studies, as well as the studies of EVANS *et al.* (7) on the interaction of erythropoietin and hormones, have renewed the interest in the effect of the hormones on erythropoiesis. The role of the CNS in this chain of reactions is not clarified, and it is of considerable interest to evaluate the relative importance of the various influences from the CNS on erythropoiesis.

Investigations on the participation of the CNS in the regulation of erythropoiesis are complicated by the fact that all procedures, be they stimulatory or ablative, introduce a series of reactions in the experimental animals. These reactions may condition the animal and secondarily influence the erythropoiesis or the parameters measured without any real change in erythropoiesis. The latter is particularly true regarding all previous work which has been performed using changes in hemoglobin, number of erythrocytes or hematocrit as the only parameters because it is well-known that great changes in plasma volume may occur with procedures influencing the hypothalamo-pituitary system.

In our hands electrical *stimulation of the hypothalamus* increased the reticulocytes and RCM in 8 of 28 stimulated rabbits (36). Increased plasma erythropoietin levels were demonstrated in 6 of 16 rabbits studied (13), indicating that the effect of hypothalamic stimulation on erythropoiesis was mediated through erythropoietin. MIRAND *et al.* (29) have recently confirmed the latter finding in monkeys. Following electrical stimulation of the hypothalamus they found increased plasma erythropoietin levels using polycythemic mice as recipients. KELL *et al.* (22) observed polycythemia following electrical stimulation of cortical loci of the prefrontal and pyriform areas in dogs and cats. In some of these animals, however, there was medial hypertrophy and intimal proliferation in pulmonary arteries suggesting that some degree of hypoxemia with secondary polycythemia may have occurred. In none of the above

Table I

he differences between the values after and before an experimental period of 2 weeks (± s.
bbits receiving electrical stimulation of the hypothalamus and rabbits injected with ACTH, GH
and GH-ACTH-thyroxin-testerone

	Control	Hypothalamic stimulation Pos. response	ACTH	GH	GH-ACTH
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ml	−0.4±0.5	3.1±0.3	1.1±0.5	0.5±1.0	0.9±0.8
in, g	0.0±0.3	−0.3±0.3	−0.2±0.3	−0.5±0.4	−0.6±0.4
tes, mill.	0.2±0.3	−0.1±0.3	−0.2±0.2	−0.4±0.2	−0.4±0.1
it, %	−0.9±1.1	3.0±0.6	0.9±0.5	−0.3±0.9	−1.2±0.9
	−4.6±4.3	7.9±3.6	3.6±1.8	4.0±2.9	3.6±2.6

Table II

ythropoietin levels in rabbits following 2 weeks of hypothalamic stimulation (positive respon
daily injections of ACTH, GH, GH ACTH and GH-ACTH-thyroxin-testosterone for 2 wee
see 13, 14, 17 18)

[illegible]	Intact mice	Starved mice		Polycythem
[illegible]	Reticulocytes	Reticulocytes	Fe^{59} uptake	Reticulocytes
sponse group	increased	increased	increased	increased
	insignificant increase		insignificant increase	
H				
H-Thyroxin-testosterone			inhibition (?)	

Discussion

The concept of a humoral regulation of erythropoiesis through erythropoietin is generally accepted but the details in the mechanism are unknown for several steps. Hypoxia is considered to be the fundamental stimulus for erythropoietin production but there are few data regarding the site of recording of the variations in oxygen tension. Although perfusion studies of the kidneys (8, 24) and experiments with clamping of the renal artery (19) suggest that both the registration of oxygen tension and the production of

pothalamus but did not consider the possible effect of pituitary hypofunction. Their results are in contrast with ours regarding the localization of the lesions within the hypothalamus, but they otherwise support the concept that hypothalamic lesions may reduce the erythropoietic response to hypoxia.

The ablation studies referred to above, except the studies of PILIERO *et al.*, indicate that hypothalamic lesions interfere with the normal erythropoietic response to hypoxia. Thus both stimulation and ablation of the hypothalamus induce marked changes in erythropoiesis. These studies provide a substantial amount of evidence that the hypothalamic area is of fundamental importance in the regulation of erythropoiesis.

The mechanism by which hypothalamus exerts its influence is, however unknown. The working hypothesis for our studies has been that a specific group of cells located in this area record the variations in oxygen tension and by nervous or humoral routes stimulate the target organ(s) to erythropoietin production. Although erythropoietin is produced following perfusion of isolated kidneys, this does not prove that the kidney is the only organ recording variations in oxygen tension in physiological conditions, and experiences with the anephric man (30) and animal (28) clearly indicate that this is not the case. Only few direct data are, however available on this point. BACIU *et al.* (1) have studied the effect of clamping the carotid artery in dogs with the contra lateral artery ligated or sutured to a transplanted kidney They also investigated the erythropoietin levels in plasma of nephrectomized rats exposed to hypoxia From these studies they concluded that erythropoietin was produced following cerebral hypoxia and not following renal hypoxia

Studies on the unit activity in the hypothalamus followin[illegible] hypoxia have been performed by CROSS AND SILVER (5) They [illegible] served that a high proportion of tested neurons in the posterior [illegible] lateral areas of the hypothalamus was excited by hypoxia [illegible] that hypoxia induced a sympathetic discharge similar to th[illegible] sulting from hypothalamic stimulation. These experiments [illegible] some evidence in favor of the presented hypothesis but [illegible] may also be interpreted as a stress reaction. Although su[illegible] indirect evidence the theory of specific oxygen receptive [illegible] trolling erythropoietin production has only vague, dir[illegible] mental support. Studies combining methods in neur[illegible]

kidney physiology and hematology will be necessary to explore this field further

There seems at present to be *three main routes by which the hypothalamic area may control erythropoiesis*, through the pituitary gland, through the autonomic nervous system or through specific nervous or humoral stimuli.

The generally accepted route is the control exerted via *the pituitary gland*. In the studies reported in this paper work has been done to compare the effect of hypothalamic stimulation with the effect of pituitary and target organ hormones. These comparative studies have revealed that GH may produce a similar reticulocytosis as hypothalamic stimulation but no increase in RCM. The combination of GH ACTH thyroxin testosterone produced polycythemia but the plasma erythropoietin levels were not elevated at the end of the experimental period as following hypothalamic stimulation, and there were signs rather of inhibition when the plasma was tested in starved mice. It may be concluded that hypothalamic stimulation and combined hormone therapy may have a similar end effect on erythropoiesis, *i. e* polycythemia. This does not prove that the operating mechanism has been the same, and, in fact, the differences in the plasma erythropoietin levels found in the two groups indicate that it is not. It must, however be concluded that it is difficult to provide conclusive evidence in favor of an extrapituitary influence on erythropoiesis following hypothalamic stimulation unless the experiments are carried out in hypophysectomized animals.

It has repeatedly been stated that the effect of the hormones on erythropoiesis is secondary to their control of oxygen consumption (6) Although this remains an important part of the control, recent studies have changed this view considerably and have indicated that the hormones have a more specific role in the regulation of erythropoiesis. Thyroxin (27) testosterone (10) GH (9 17 33) and prolactin (21) have been found to stimulate erythropoiesis and erythropoietin production or interact with erythropoietin in an additive manner (7) The studies by JEPSON AND LOWENSTEIN (21) and in this laboratory* on the effect of ovine prolactin on Fe^{59} erythrocyte uptake in polycythemic mice may further indicate a direct effect of pituitary hormones on erythropoiesis, at least in

* Unpublished data

some species. These findings point to possible routes by which hypothalamus may exert its control of erythropoiesis unrelated to variations in oxygen consumption.

Another possible route of hypothalamic control of erythropoiesis is through *the autonomic nervous system*. Stimulation or ablation of hypothalamic structures interfere with the integrity of this system. This may influence the cardio-pulmonary function and possibly induce hypoxemia by this mechanism, a possibility which is relevant in the interpretation of the data reported by KELL *et al.* (22) In the stimulation experiments reported in this paper respiration and blood pressure were recorded in four of the animals showing a positive erythropoietic response, and there was no significant difference before and after stimulation. Of more direct concern in several experiments is the question of changes in vascular tonus or blood flow following stimulation or ablation of the hypothalamus, section of the spinal cord or resection of sympathetic or parasympathetic nerves. In view of recent evidence for erythropoietin production by the kidneys (8, 24 31) it is possible that the reduced response to hypoxia following such procedures or the effect of hypothalamic stimulation, may be due to changes in renal circulation.

Although most information concerning the control of renal circulation support the view of autoregulation (41) other data indicate that renal circulation is extremely sensitive to the catecholamines (41) Electrical stimulation of the splanchnic nerves causes severe, but transient renal vasoconstriction (40) GÖMÖRI *et al* (12) report data indicating both a cerebral and local regulatory mechanism for the control of renal circulation. If their findings are confirmed, both renal and cerebral hypoxia may lead to changes in renal circulation which possibly may be the basis for variations in erythropoietin production. This view opens the possibility for a uniform hypothesis regarding erythropoiesis regulation *and is a working hypothesis which may stimulate to further experiments.*

The studies of TAKAKU *et al.* (39) on the effect on erythropoietin production of bilateral splanchnic nerve resection lend some support to such a hypothesis while the studies of BACIU *et al.* (1) on transplanted kidneys in the dog and other studies on transplanted kidneys in the human do not support the theory

Common to many studies on the influence of the CNS on erythropoiesis has been that the reticulocyte counts have shown the most marked deviations from the normal. This raises the question whether the reticulocyte response to such procedures could be the result of interference with the reticulocyte release mechanism. The intermediate links in this control are unknown, but the finding of reticulocyte increase following cholinergic stimulation (35) may indicate that the autonomic nervous system participates in the control. It is not likely however that reticulocyte release only was the cause of the reticulocytosis in our stimulation experiments because the increase was slow and reached a maximum after more than three days, and because a concomitant rise in RCM and plasma erythropoietin was found.

The concept of specific groups of cells in the hypothalamus recording variations in oxygen tension and regulating erythropoiesis through *specific nervous or humoral stimuli* has been the basis *for the present work. The data presented in this paper fit well with* and are readily explained by such a hypothesis. The finding of elevated plasma erythropoietin following hypothalamic stimulation (13 29) indicate that the erythropoietin production is subject to cerebral control. The reduced erythropoietic response to hypoxia following hypothalamic ablation (16 26) cord section (37) and splanchnic nerve resection (39) may point to the nervous system as mediator for the stimulus to the target organ. The data provide, however only indirect evidence, and it must be concluded that there is no entirely *conclusive* evidence in favor of this hypothesis. Progress in research in this field, particularly regarding the hormones, has made the terms specific non specific effects of less value and significance, and they should probably be abandoned in this connection.

Although the mechanism by which CNS controls erythropoiesis is not clarified, the data presented in this paper and referred to strongly support the concept that the CNS is of fundamental importance in the regulation of erythropoiesis. Future studies using perfusion techniques in hypophysectomized and/or nephrectomized animals with simultaneous recording of arterial oxygen saturation, oxygen tension and blood flow in the organs, may give more clear cut answers to the question of how the CNS exerts its influence on erythropoiesis.

Summary

Previous and recent data indicate that the central nervous system participates in the regulation of erythropoiesis. The present paper reports studies on the hematological changes following hypothalamic stimulation and on comparative hormone experiments. These studies strongly support that the hypothalamus is of fundamental importance in the regulation of erythropoiesis. The mechanism by which the central nervous system exerts its influence is unknown but there are at present three main possibilities; an effect through the pituitary gland, through the autonomic nervous system or directly on erythropoietin production.

Résumé

Les résultats d'études antérieures et récentes indiquent que le système nerveux central participe à la régulation de l'érythropoïèse. Le travail présent rapporte les études des altérations hématologiques causées par la stimulation de l'hypothalamus et des expériences comparées faites avec des hormones. Ces études confirment que l'hypothalamus une importance fondamentale dans la régulation de l'érythropoïèse Le mécanisme par lequel l'hypothalamus exerce cette influence est inconnu. Les trois possibilités principales sont les suivantes une influence par l'intermédiaire de l'hypophyse ou du système nerveux autonome ou encore une influence directe sur la production d'érythropoïétine.

Zusammenfassung

Frühere und neuere Ergebnisse sprechen dafür daß das Zentralnervensystem bei der Regulation der Erythropoese eine Rolle spielt. In der vorliegenden Mitteilung wird über hämatologische Veränderungen nach Reizung des Hypothalamus und über vergleichende Versuche mit Hormonen berichtet. Die Untersuchungen bestätigen, daß dem Hypothalamus eine grundlegende Bedeutung bei der Regulation der Erythropoese zukommt. Der Mechanismus dieser zentralnervösen Steuerung ist unbekannt. Zur Zeit ergeben sich dafür drei Möglichkeiten eine Wirkung über die Hypophyse, über das autonome Nervensystem oder direkt auf die Produktion von Erythropoietin.

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Iron Absorption and Excretion in Aregenerative Anaemia

A Collaborative Swedish-American Whole Body Counter Study*

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It is known that the intestinal absorption of iron is influenced not only by the body iron stores (2, 6 16, 19) but also by the level of erythropoietic activity (2 3 14 28) Thus, hypertransfusion (3 28) may reduce and increased erythropoiesis (2, 3) increase the absorption. Whether information about the level of the stores and/or of the bone marrow activity is transmitted to the intestinal cell via the intracellular iron concentration and apoferritin saturation (5 8 9) the plasma transferrin concentration (18, 27) the plasma iron turnover rate (29) the blood haemoglobin concentration (15 24) or specific humoral factors (1 7 12) is not known. The purpose of the present study is to examine the relation between intestinal iron absorption and these other parameters in humans with reduced erythropoetic activity (aregenerative anaemia)

The rate of iron excretion has been studied less. Both in conditions with increased (10 11 21) and in conditions with decreased iron stores (19 21 22) the rate of excretion appeared to be reduced.

Because of the relative scarcity of patients with aregenerative anaemia in areas where whole body counters are available, the present study was carried out as a joint venture between the Swedish and American laboratories.

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Table I
General description of patients with aregenerative anaemia.

Patient	Sex/Age	Diagnosis	Duration of disease (years)	Total transfusions	Remarks
AF	M/67	AA Pancytopenia	1.5	82	Late leukemoid blood picture, *autopsy only AA*
AJ	M/40	AA, Pancytopenia	1.5	37	Splenomegaly
SH	M/62	Myelosclerosis Pancytopenia	6	55	Previous polycythemia, splenectomy
MB	F/74	Myelofibrosis	8	1	Splenomegaly benign course
AL	F/60	Myelofibrosis Myelosclerosis	?	6	Pulmonary TBC splenomegaly
GM	M/78	AA	?	33	Benign course
SC	F/68	AA, Carcinoma of colon	5	15	Tumor resection 4 years before anaemia hepatic metastases at autopsy
AR	M/70	AA	2	16	Insecticide exposure
VE	M/41	AA, Pancytopenia	6	50	Unusual reticulo endotheliosis. Myeloproliferative at end of course
BL	M/24	AA, Pancytopenia	2	40	Many infections
TR	M/53	AA	2	15	Proerythroblastic maturation arrest
MA	M/60	AA Pancytopenia	1.5	40	Benign course
ST	F/5	AA, Fanconi' anaemia, pancytopenia	5	2	Absence of thumbs, microcephaly renal anomalies
ZI	F/47	Myeloid metaplasia, myelofibrosis	5	200	Splenectomy
FR	M/67	AA	2.5	150	Leukemoid peripheral blood picture
FE	F/64	AA, Pancytopenia	3.5	200	Benign course
McC	F/12	AA, Pancytopenia	12	90	Congenital hypoplastic anaemia

AA = Aregenerative anaemia
Approximate number

Material

It is recognized that number of diseases of varying etiology pathologic and clinical pictures can be characterized by an aregenerative anaemia. Aregenerative anaemia is here defined as an anaemia, not mainly due to bleeding or haemolysis or to deficiency of iron, vitamin B_{12} or folic acid, with subnormal reticulocyte response, subnormal Fe^{59} incorporation into erythrocytes, and refractory to treatment other than transfusions. Only patients without known primary diseases (renal, leucemic etc.) were included. *Red cell Fe^{59} incorporation* is normally 70–90% of the tracer dose (4). In all our patients so studied, the results were subnormal–ranging from 0–55%.

The 17 patients are described in Tables I and II. Cases A. F to G. M. were studied at Karolinska and S. C. to McC. at Brookhaven.

Normal Fe^{59} absorption was studied in Stockholm in 20 male volunteers. At Brookhaven 9 male and 7 normal postmenopausal females were studied. Normal Fe^{59} excretion was studied in Stockholm in 10 volunteer men or menopausal females, at Brookhaven in 9 persons.

Table II

Selected erythrocytic data of patients with aregenerative anaemia.

Patient	Haemoglobin gm %	Total Reticulocytes per mm³	Bone marrow: Cellularity sections	Bone marrow: Fibrosis, sections	Bone marrow: Iron stain, aspirate
AF	4.0	0–21.000	a. NC	—	moderate
			b. HRC	—	
AJ	7.3	0–117.000	NC	+	increased
SH	8.1	94.000–193.000	a. HRC	+	
			b. HOC	+	
MB	7 1	48.000–190.000		+	
AL	8.6	22.000–62.000	HRC	+	small
GM	9.1	25.000	HOC	—	moderate
SQ	6.7	16.640	NC	—	increased
AR	8.6	5.800	HOC	—	
VE	8.2	0	HRC	—	increased
BL	7 4	3460	HOC	+	increased
TR	7.8	0			increased
MA	5.7	50.880			increased
ST	4.1	59.590			small
ZT	11 4	19 150	HOC	+	increased
FR	6.0	0			increased
F	5.8	0			increased
McC	5.0	0			increased

Methods

Iron administration. 250 μg Fe^{59} in the form of ferrous sulfat or ferrous citrate were administered orally to fasting patients. Stockholm patients received 1 3 μg Fe^{59} Brookhaven patients 1 5 μc.

Because iron absorption was frequently low neither turnover nor red cell Fe^{59} incorporation could always be studied adequately on the basis of the absorbed iron. All Karolinska patients and 5 of the Brookhaven patients therefore received 1 10 μg Fe^{59} in the form of plasma bound ferrous citrate intravenously when the absorption study was completed.

Absorption measurements: The principle of studying absorption with the help of whole body counting has been described (19 21). The total body radioactivity was measured initially after oral administration and also 10 to 21 days later when all unabsorbed iron had been excreted with the faeces.

Two different whole body counters were used. Both the one in Stockholm (13, 13a) and the one at Brookhaven (19, 20, 21) have been described in detail. In principle,

Table II (Continued)

(NO = Normal cellularity HOC = Hypocellular HRC = Hypercellular)

Serum iron mg/100 ml	UIBC mg/100 ml	Iron absorption per cent	Iron excretion % per day	Months followed	Erythrocyte Fe^{59} incorp. per cent	Erythrocyte life span days	Plasma Fe^{59} T ½ minutes
207	100	0.20	0		0	36	
				4.5			
207	42	0.01	0		29–33	33	
210	125–205	10.20	0.085	6.5	23–26	32	
				7			
19	200	1.05	0.078		6–30	69	
				4.5			
56–123	110	3.95	0.156		40–55	43	
				3.5			
159–208	*251 160*	*1.5.*	*0.199*		33–45	*4*[illegible]	
				5			
138	115	9.00			12.8		
		8.20	0.173		0.3		
				5			
225	0	6.40	0.109		3.5		
				7			
360	28	2.90					
169	23	29.00	0.177		0.4		190
				6.5			
238	135	10.20			22.0		307
247	144	29.80					
160	0	2.30	0.150		2.1		68
				4			
176	50	5.00			0		
242	30	3.10			0		270
214	30	2.00			23.0		275

each consisted of a chair for the patient, a steel shield to reduce background activity a large stationary NaI (Tl) crystal detector and a multichannel analyzer.

Excretion measurements: The loss of absorbed (patients AR and VE) or injected Fe^{59} was followed with the Brookhaven whole body counter for 4 to 7 months (average 170 days) and in Stockholm for 3½ to 7 months (average 150 days). The daily percentage loss (regression of the log Fe^{59}-content on time x 1/log e) was calculated on an IBM 1401 computer (Stockholm) or Marlin Digital computer (Brookhaven).

Measurement of Fe^{59}-uptake by red cell mass. Radioactivity of whole blood samples was measured in well-type NaI (Tl) crystal detectors 1, 2 and 3 weeks after the oral or parenteral administration of iron. Cell counts and haemoglobin concentrations were determined in the customary manner.

At Brookhaven, circulating red cell mass was determined by labeling red cells with Cr^{51} (patients MA, ZI, SC and McC). In Stockholm, total body haemoglobin was determined in all patients with the alveolar carbon monoxide method of Sjöstrand (26).

Bone marrow punctures and smears were obtained by customary methods. It was not considered possible to judge bone marrow cellularity from smears alone. In 4 of the Stockholm patients (AF, AJ, AL and GM) sections of bone marrow particles collected

Table III
Iron absorption and excretion.

	Normal controls		Aregenerative anaemia	
group	Iron absorption[1]	Iron excretion[2]	Iron absorption	Iron
[illegible]holm	18.1 ± 2.00 (20)	0.252 ± 0.078 (7)	2.62 ± 1.58 (6)	0.066 ±
[illegible]haven	16.4 ± 1.41 (16)	0.150 ± 0.012 (9)	9.80 ± 3.03 (11)	0.150 ±
	17.3 ± 1.26 (36)	0.186 ± 0.035 (16)	7.36 ± 2.18 (17)	0.112 ±

% of oral dose (0.25 mg Fe^{++}) Mean ± S. E.
% of total body-radio iron per day Mean ± S. E.
Figures in brackets indicate No. of subjects.

on watch glass were studied for fibrosis or sclerosis, the amount of stainable non-hemin iron, and the relative amounts of fat cells and bone marrow cells. At Brookhaven bone marrow sections were obtained at biopsy or autopsy in 5 of the patients.

Unsaturated iron binding capacity (UIBC) was measured by the colorimetric method (25). Serum iron and UIBC-determinations for Brookhaven patients were first performed in collaboration with the Haematology Department of Mt. Sinai Hospital, and then by a modified Schade's technique at Brookhaven.

The red cell life span was estimated in Stockholm by measuring the endogenous carbon monoxide production according to SJÖSTRAND (26). Statistical calculations were performed on an IBM 1401 computer

Results

Iron absorption. The values are given in Table III The mean absorption in the patients with aregenerative anaemia is less than half of that in the controls. The difference is statistically significant.

In patients with one iron absorption-regulator (erythropoiesis) out of function, one could have hoped to find a good correlation between the other regulator (body iron stores) and absorption itself. Such a relationship is suggested by Fig 1 indicating that the patients having recieved the most transfusions absorb the least iron. (Correlation coefficient r = −0.358 regression coefficient b = −2.584 standard deviation of b SDb = 1 74) Similarly the patients with increased iron concentration in the bone marrow absorbed on an average 6.6 / of the oral radioiron, as compared to the 9.3 / absorbed by those with small or moderate amounts of bone marrow iron. Statistically however these findings are not significant.

A relation between the unsaturated transferrin level in the plasma and iron absorption has been suggested by HALLBERG and his group (27) but could not be confirmed initially (18, 21) Nor was there any significant correlation in the present material, either between the UIBC (r = 0.089 b = 0 66 SDb = 1 99) or between

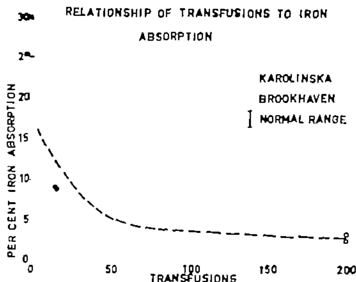


Fig 1 Relationship of transfusions to iron absorption. Statistically the suggested decrease in absorption with an increasing number of transfusions is not significant.

the serum iron (r = 0 093 b = 0 720 SDb = 2.069) and the absorption.

A relation between the haemoglobin concentration and the iron absorption is suggested by Table IV but the coefficient of regression for the iron absorption upon the haemoglobin concentration does not differ from zero in a statistically significant manner. Later studies, however where these and other patients were transfused and the iron absorption studied again when the haemoglobin values were normalized, confirm this relation (24)

The *iron excretion* is a multiple exponential function which was approximated as a single exponential in Tables II and III. The percentage excretion figures thus obtained are only relative, and do not indicate the loss of unlabeled iron.

A seemingly significant decrease in iron excretion was found in the patients with aregenerative anaemia, but the unexplained difference between Stockholm and Brookhaven groups in this respect makes this result subject to cautious interpretation.

Other results Thirteen out of 17 patients studied had hyperundermia, (mean 0.196 mg Fe/100 ml SD 0 071) and 14 out of 17 had low UIBC values (mean 0 085 mg/100 ml, SD 0 068) In all 6 patients, where the carbon monoxide haemoglobin concentration

Table IV

Relationship of iron absorption and hemoglobin at time of iron absorption

	Hemoglobin 4–5.9 gm %	Hemoglobin 6–7.9 gm %	Hemoglobin over 8 gm %
Iron absorptions	0.2	0.01	10.2
	10.2	1.05	3.95
	29.8	9.0	1.52
	3.1	2.9	8.2
	2.0	29.0	6.4
		5.0	2.3
Average	9.1	7.8	5.4

Statistically the suggested relationship is not significant.

was determined, a somewhat elevated level of COHb was found. Serum haptoglobin concentrations were normal in the 5 patients where they were studied.

A positive ($r = 0.689$ $b = 0.065$ $SDb = 0.018$) and statistically significant ($0.005 > p > 0.001$) relation was found between the UIBC and the total reticulocyte count, and also between the UIBC and the erythrocyte radio-iron uptake ($r = 0.56$, $b = 2.19$ $SDb = 0.91$ $0.05 > p > 0.025$) These results suggest simply that the more efficient erythropoiesis is, the higher are reticulocyte counts and erythrocyte iron uptake, and the more rapidly is iron removed from the plasma with an increasing UIBC as a result. In agreement with this reasoning, a *negative* correlation was found between the serum iron concentration on the one hand and the total reticulocyte count ($r = -0.45$) and the erythrocyte iron uptake ($r = -0.27$) on the other These latter correlations however were not significant statistically

Some expected and unexciting correlations were also found, such as between the reticulocytes and the erythrocyte iron uptake ($r = 0.51$) and the serumiron and the UIBC ($r = -0.36$)

Discussion

Method. The redistribution within the body of orally administered Fe^{59} will cause changes in the geometry even if the relative positions of crystal and patient are constant. An error is thus introduced into the absorption measurements (23) This error was not more pronounced for the Stockholm counter than for the more heavily shielded Brookhaven counter A detailed analysis of the

magnitude of these errors in the Stockholm counter including regressions of body counter values on those obtained with the fecal recovery method or the red blood cell uptake method has been published separately (13)

It is known that the normal human intestinal passage time averages 5 days. However even 10 days after the oral administration of Fe^{59} radioactivity excretion is much more rapid than later on (22) This has been attributed to the desquamation of intestinal mucosa cells which have taken up Fe^{59} Whether this iron should be regarded as absorbed or not is a matter of semantics. In the present study iron retained after 10–21 days was regarded as absorbed.

Absorption. If a wide range of intracellular irons, UIBC:s, serum irons, plasma iron turnover rates, and haemoglobin values had been found in our patients, then the relative importance of these possible intermediate factors in the regulation mechanism could have been better studied. Under the present conditions, it is only possible to state that patients with aregenerative anaemia, most of whom had high intracellular irons, low UIBC:s, high serum irons and slow plasma iron turnover rates, have a significantly decreased iron absorption. Only a systematic comparison with other clinical groups, where different values are found, will make it possible to single out the most important of the regulating factors.

The possible importance of the haemoglobin concentration for the iron absorption will be discussed elsewhere (24)

Excretion. It is difficult to interpret the excretion values except in connection with a complete kinetic analysis of human iron metabolism. Less of the radio-iron is found in the erythrocytes and more in other tissues in aregenerative anaemia than in normal controls. Intracellular iron in other tissues is increased. Both factors would be expected to increase the rate of excretion, yet no such increase was found. This suggests that the turnover of non-haemoglobin iron is slow even compared to the very slow turnover of erythrocyte iron during the first 4 months following injection.

Serum iron. The significant correlation between parameters related to the intensity of erythropoiesis (reticulocytes and erythrocyte iron uptake) and the serumiron and UIBC emphasize the importance of bone marrow iron uptake in regulating the serum iron metabolism.

Summary

Of 17 patients with aregenerative anaemia 13 had hypersideremia and 14 low unsaturated iron binding capacity. The more depressed erythropoiesis was, the higher was the serumiron and the lower the UIBC. The average iron absorption was significantly lower in patients with aregenerative anaemia than in normal individuals. The most anemic patients seemed to absorb more than those with less anaemia and the more transfusions the patients had received, the less iron they seemed to absorb. These relations were not statistically significant. Although large amounts of iron had been given to these patients in the form of transfusions, and although they had hypersideremia and, often, increased iron amounts in the bone marrow, the rate of iron excretion was not increased.

Résumé

Parmi 17 malades atteints d'anémie arégénératrice, 13 avaient une hypersidérémie et 14 une diminution de la capacité latente de saturation en fer. Plus l'érythropoïèse était réduite, plus le taux de fer sérique était haut et plus la capacité latente de saturation en fer était faible. La résorption moyenne de fer était chez les malades atteints d'anémie arégénératrice de façon significative moindre que chez des personnes saines. Les malades avec une anémie avancée semblaient résorber plus de fer que ceux avec une anémie faible, et plus ils avaient reçu de transfusions, moins ils semblaient résorber de fer. Ces relations n'étaient pourtant pas statistiquement significatives. Quoique ces malades aient reçu de grandes quantités de fer sous forme de transfusions et quoiqu'ils présentaient une hypersidérémie et des quantités augmentées de fer dans la moelle osseuse, leur excrétion de fer n'était pas accélérée.

Zusammenfassung

Von 17 Patienten mit aregeneratorischer Anämie hatten 13 eine Hypersiderinämie und 14 eine Verminderung der ungesättigten Eisenbindungskapazität. Je stärker die Erythropoese herabgesetzt war, umso höher war das Serumeisen und umso niedriger die ungesättigte Eisenbindungskapazität. Die mittlere Eisenresorption war bei Patienten mit aregeneratorischer Anämie signifikant geringer als bei Gesunden. Patienten mit fortgeschrittener Anämie schienen mehr Eisen zu resorbieren als solche mit geringerer Anämie, und je mehr Transfusionen sie erhalten hatten, umso weniger Eisen schienen sie zu resorbieren. Diese Beziehungen waren jedoch statistisch nicht signifikant. Trotzdem diese Patienten grosse Mengen von Eisen in Form von Transfusionen erhalten hatten und obgleich sie eine Hypersiderinämie und erhöhte Eisenmengen im Knochenmark aufwiesen, war ihre Eisenausscheidung nicht vermehrt.

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The Activation of Fibrinolysis with 3-Pyridil Acetic Acid

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and M. Verstraete

The activation of fibrinolysis by nicotinic acid is well known (17 11) The molecular structure of 3-pyridil-acetic acid (3-PAA) is closely related to that of nicotinic acid (Fig 1)

Activation of fibrinolysis by 3-PAA was first described by Antonini *et al.* (4) and Dettori *et al.* (6) who observed shortened clot lysis times in approximately one third of the patients who were given 3-PAA per os. Introzzi *et al.* (9) observed a significant increase of plasmin and plasminogen after one administration of 500 mg 3-PAA intravenously Lombardi *et al.* (10) confirmed that increase of fibrinolytic activity may occur after administration of 3-PAA per os. Gibelli *et al.* (8) found that one intramuscular administration of 150 mg 3-PAA resulted in a significant increase of the plasminogen level and of plasmin activity between 20 and 60 minutes after administration. Repeated intramuscular injection of 150 mg 3-PAA daily during 16 consecutive days provoked a significant rise of plasmin activity the first day only but a gradual increase of the plasminogen level over the sixteen day experimental period.

In this study we examined the effect of a single and of repeated intravenous injections of 3-PAA on fibrinolysis, the plasminogen level, the level of some clotting factors and on the venous leucocyte population.

Methods and Materials

3-pyridil acetic acid was supplied in liberal amounts as 'Lioscor'® by Lepetit S. p. a., Milano; vials for intravenous administration, containing either 150 mg or 500 mg 3-PAA, were delivered.

Fibrinolytic evaluations, comprising the determination of the lysis time of dilutions of whole blood, the fibrin plate technique, and the preparation of plasma and euglobulin fraction were performed as described by Amery *et al.* (1).

Thrombotest times (12) were determined on whole citrated blood.

One-stage prothrombin times were performed using Difco rabbit brain Bacto-Thromboplastin.

P- and P-assay was performed as described by Owren and Aas (13). Factor V was determined according to Stormorken (14).

Stypven times were obtained by substituting Russel viper venom for brain Thromboplastin in the P and P-assay.

Thrombin times were measured as described by Vermylen and Verstraete (16).

Fibrinogen was determined on whole citrated blood with the FPT-test, as described by Vermylen et al. (15).

Leucocyte counts were obtained using Turk's dilution fluid and Bürker's slide.

Plasminogen was assayed, using either a standard bovine clot test system or a caseinolytic method, according to De Vreker (7). Statistical methods were adopted from Bailey (5).

Experimental procedure: At 5 p. m. blood sample A was drawn. At the same moment, 3-PAA was injected intravenously. Fifteen minutes later blood sample B was taken, and a slow intravenous infusion of glucose 5% was installed. At 9.00 p. m., 1 a. m. and 5 a. m., 3-PAA was injected into the intravenous lead. At 8 a. m. blood sample C was drawn and 3-PAA injected. At 8.15 a. m. blood sample D was taken. In the first series of 27 experiments, 150 mg 3-PAA were injected repeatedly. In the second series of 8 experiments, repeated injections of 500 mg 3-PAA were made.

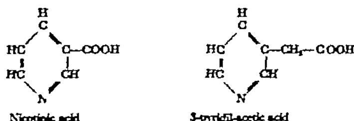


Fig. 1 Molecular structure of 3-pyridil-acetic acid and nicotinic acid.

Results

A. Alteration of whole blood, plasma and euglobulin fibrinolytic activity by repeated intravenous injections of 3-PAA

1 The alteration of the fibrinolytic activity of whole blood following repeated intravenous injections of 3-PAA was studied by determining the lysis times of clots made from serial dilutions of whole blood. With blood diluted 1 : 4 the test was found to be most sensitive for detecting increased fibrinolytic activity. Table I gives the distribution of the lysis times of blood, diluted 1 : 4, taken before and after a first and fifth injection of 150 mg 3-PAA.

As shown, no evidence of increased whole blood fibrinolytic activity was observed in the fifteen persons before the first injection of 150 mg 3-PAA. After the first injection, an important increase

Table I

Distribution of the lysis times of the blood samples diluted 1 : 4 (15 experiments).

	Number of experiments with lysis time					
Blood sample	0–9 h	10–19 h	20–29 h	30–39 h	40–49 h	50 h
A	—	—	—	—	1	14
B	7	1	2	1	—	4
C	—	—	—	—	—	15
D	—	—	—	—	—	15

Table II

Distribution of the surfaces lysed on unheated fibrin film by the different plasma samples (24 experiments with repeated injection of 150 mg 3-PAA)

	Number of experiments giving lysed surface of				
Plasma sample	0 mm²	1–50 mm²	51–100 mm²	101–200 mm²	201–400 mm²
A	24	—	—	—	—
B	16	2	2	2	2
C	24	—	—	—	—
D	24	—	—	—	—

Table III

Distribution of the surfaces lysed on heated fibrin film by the different plasma samples (24 experiments with repeated injection of 150 mg 3-PAA).

	Number of experiments giving lysed surface of				
Plasma sample	0 mm²	1–50 mm²	51–100 mm²	101–200 mm²	201–400 mm²
A	24	—	—	—	—
B	12	8	3	1	—
C	24	—	—	—	—
D	24	—	—	—	—

of whole blood fibrinolytic activity (lysis time 0–9 h) is observed in 7, a moderate increase (lysis time 10–39 h) in 4 persons. No increased whole blood fibrinolytic activity is noted either before or after the fifth injection of 3-PAA.

2. The alteration of plasma fibrinolytic activity after repeated intravenous injections of 3-PAA was measured on unheated and heated fibrin film. The distribution of the surfaces of fibrin lysed by plasmata obtained before and after a first and fifth intravenous injection of 150 or 300 mg 3-PAA is summarized in Tables II–V.

It is observed that plasma fibrinolytic activity is increased in 8/24 persons on unheated and in 12/24 persons on heated fibrin film after a first intravenous injection of 150 mg 3-PAA. A first

Thrombotest times (12) were determined on whole citrated blood.

One-stage prothrombin times were performed using Difco rabbit brain Bacto-Thromboplastin.

P and P-assay was performed as described by Owren and Aas (13). Factor V was determined according to Stormorken (14).

Stypven times were obtained by substituting Russel viper venom for brain Thromboplastin in the P and P-assay

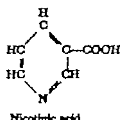
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Plasminogen was assayed, using either standard bovine clot test system or a caseinolytic method, according to De Vreker (7) Statistical methods were adopted from Bailey (3)

Experimental procedure: At 5 p. m. blood sample A was drawn. At the same moment, 3-PAA was injected intravenously Fifteen minutes later blood sample B was taken, and slow intravenous infusion of glucose 5% was installed. At 9.00 p. m. 1 a. m. and 5 a. m., 3-PAA was injected into the intravenous lead. At 8 a. m. blood sample C was drawn and 3-PAA injected. At 8.15 a. m. blood sample D was taken. In the first series of 27 experiments, 150 mg 3-PAA were injected repeatedly In the second series of 8 experiments, repeated injections of 500 mg 3-PAA were made.



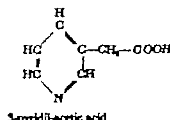


Fig 1 Molecular structure of 3-pyridil-acetic acid and nicotinic acid.

Results

A. Alteration of whole blood, plasma and euglobulin fibrinolytic activity by repeated intravenous injections of 3-PAA

1 The alteration of the fibrinolytic activity of whole blood following repeated intravenous injections of 3-PAA was studied by determining the lysis times of clots made from serial dilutions of whole blood. With blood diluted 1 4 the test was found to be most sensitive for detecting increased fibrinolytic activity Table I gives the distribution of the lysis times of blood, diluted 1 4 taken before and after a first and fifth injection of 150 mg 3-PAA.

As shown, no evidence of increased whole blood fibrinolytic activity was observed in the fifteen persons before the first injection of 150 mg 3-PAA. After the first injection, an important increase

3 The alteration of euglobulin fibrinolytic activity after repeated intravenous injections of 3-PAA was measured on unheated and heated fibrin film. The mean values of the surfaces of fibrin lysed before and after a first and fifth intravenous injection of 150 or 500 mg 3-PAA are given in Table VI.

From statistical calculations on individual paired samples it appeared that with 150 mg 3-PAA the increase of euglobulin fibrinolytic activity after a first injection was significant ($P < 0.001$ on unheated fibrin, $0.01 > P > 0.002$ on heated fibrin) after a fifth injection no variation of euglobulin activity was found ($P > 0.1$ on both substrates) The euglobulin fibrinolytic activity before a fifth injection did not differ significantly from the activity observed before the first injection of 150 mg 3-PAA.

With 500 mg 3-PAA the increase of euglobulin fibrinolytic activity after a first injection was significant ($0.02 > P > 0.01$ on unheated fibrin film, $0.05 > P > 0.02$ on heated fibrin film) after a fifth injection no variation of euglobulin fibrinolytic activity was found ($P > 0.10$ on unheated and heated fibrin film)

From Table VI it seemed apparent that the increase of euglobulin activity was more pronounced after a first injection of 500 mg than after administration of 150 mg 3-PAA. Statistical comparison of the mean euglobulin activity on unheated fibrin film after a first injection of 150 mg and after 500 mg 3-PAA showed the probability that both means would be equal to be 0.01–0.02. On heated fibrin film however this value is larger than 0.10

Table VI suggests that after repeated injection of 500 mg 3-PAA the fibrinolytic activity of the euglobulins falls below the initial value. Statistical comparison of the mean euglobulin activity on unheated fibrin in sample A and in samples C and D shows a probability of equality of 0.01–0.002.

B Alteration of blood clotting factors by repeated intravenous injections of 3-PAA

Table VII summarizes the variations of the Thrombotest values, prothrombin times, the P and P assays, factor V assays, Stypven times and thrombin times after repeated intravenous injections of 150 mg 3-PAA.

As Table VII shows, the results of these coagulation tests were not modified by repeated injection of 150 mg 3-PAA intravenously

Table VI

Mean euglobulin fibrinolytic activity on unheated and heated fibrin in the different samples.

Amount 3-PAA administered	Number of exp.	Surface lysed (in mm²) by euglobulins of Sample A on UFF	Sample A on HFF	Sample B on UFF	Sample B on HFF	Sample C on UFF	Sample C on HFF	Sample D on UFF	Sample D on HFF
a. 150 mg	20	155	55	332	77	171	48	171	32
b. 500 mg	6	155	47	382	78	119	37	112	41

Table VII

Mean results of several coagulation tests performed on the different samples (in sec).

Coagulation test	Number of exp.	Normal value	Value obtained in samples A	B	C	D
Thrombotest	18	46	45	46	48	47
Prothrombin time	18	12	12	12	12	12
P-and P-assay	18	21	21	21	22	22
Factor V assay	18	22	21	21	21	23
Stypven time	18	25	24	25	26	27
Thrombin time	18	18	19	18	18	18

Table VIII

Mean fibrinogen level in the different samples.

Amount of 3-PAA repeatedly injected	N° of exp.	Fibrinogen level in mg % Sample A	Sample B	Sample C	Sample C
150 mg	18	199.3	189.4	193.9	192.0
500 mg	6	196.7	200.8	219.8	238.8

The alteration of the fibrinogen level by repeated intravenous injections of both 150 mg and 500 mg 3-PAA was studied. The mean fibrinogen values in the different samples are given in Table VIII

The significance of the observed alterations was evaluated statistically It was found that the slight decrease of the fibrinogen level after the first injection of 150 mg 3 PAA was significant at the 5 % level (0 05 > P > 0.02) The fifth injection of 150 mg 3-PAA caused no significant variation of the fibrinogen level. After five intravenous injections of 150 mg 3-PAA the fibrinogen level did not differ significantly from the pre-injection value.

No significant variation of the fibrinogen level was observed after the first intravenous injection of 500 mg 3-PAA the slight increase caused by the fifth injection is borderline significant (0 10 > P > 0 05) The fibrinogen level in samples C and D is significantly elevated (0 01 > P > 0.002) compared to the pre injection value.

Table IX

Mean plasminogen level (units per ml) in the different samples tested.

Amount 3 PAA administered	Number of experiments	Plasma sample A	B	C	D
150 mg	17	8.512	8.594	8.974	9.339
500 mg	7	287	272	270	268

Table X

Mean leucocyte content (number per mm³) of the different blood samples.

Amount 3 PAA administered	Number of experiments	Blood sample A	B	C	D
150 mg	16	7 718	5 618	7.344	6.881
500 mg	6	6.780	4 870	5.350	5.670

C The variation of the plasminogen level following repeated intravenous injections of 3-PAA

Using the standard bovine clot test system, the modification of the plasminogen level after repeated intravenous injections of 150 mg 3-PAA was measured. The same was done after repeated intravenous injections of 500 mg 3-PAA, using the caseinolytic assay technique The mean values in the different plasma samples are summarized in Table IX.

Statistical calculations on individual paired samples showed no significant difference of the plasminogen content (samples 1 and 2, 1 and 3 1 and 4 or 3 and 4 when compared had a probability of equality greater than 0 10) after repeated intravenous injection of either 150 or 500 mg 3-PAA.

D Variation of the leucocyte population following repeated intravenous administration of 3-PAA

1 Variation of the total leucocyte count Table X summarizes the mean variation of the total leucocyte count following repeated intravenous administration of either 150 mg or 500 mg 3-PAA.

Using the method of paired comparisons, the decrease of the total leucocyte count after a first intravenous injection of 150 mg or 500 mg 3-PAA was found to be highly significant ($P < 0\ 001$ after 150 mg $0\ 01 > P > 0\ 002$ after 500 mg) No significant variation of the leucocyte level was observed after a fifth intra venous administration of 150 or 500 mg 3-PAA. The leucocyte level before the fifth injection of 150 or 500 mg 3-PAA did not differ

Table XI

Mean number of neutrophile granulocytes, of lymphocytes and of monocytes per mm³ following repeated intravenous administration of 500 mg 3-PAA.

Leucocytes determined	Number of exp.	Blood sample A	B	C	D
Neutrophile granulocyte	5	4.566	2.410	3.168	3.935
Lymphocytes	5	2.031	2.328	1 770	1.540
Monocytes	5	336	298	307	319

significantly from the value observed before the first 3-PAA injection.

2 *Qualitative changes of the leucocyte population* The alteration of the number of neutrophile granulocytes, of lymphocytes and of monocytes was studied in five persons, repeatedly administered 500 mg 3-PAA intravenously The mean variations are given in Table XI

Statistical calculations on individual paired samples showed a significant drop of the neutrophile granulocyte level after a first intravenous injection of 500 mg 3-PAA (0 01 > P > 0 002) no change after a fifth injection (P > 0 10) and no difference between the pre injection level and the level before the fifth 3-PAA injection (0.10 > P > 0 05) No significant change of the lymphocyte or monocyte population was observed.

Discussion

The increase of whole blood, plasma and euglobulin fibrinolytic activity after a first intravenous injection of 3 PAA, previously described has been confirmed this increase was no longer observed after a fifth injection of 3-PAA.

The enhancement of plasma and euglobulin fibrinolytic activity was much more pronounced after a first injection of 500 mg 3-PAA than after a first intravenous administration of 150 mg 3 PAA. Euglobulin fibrinolytic activity on unheated fibrin, measured before and after the fifth injection of 500 mg 3-PAA, was significantly lower than the pre injection value A similar decrease of spontaneous activity has been observed previously in our laboratory (2) after repeated intravenous injection of a nicotinic acid compound (3-[methyl-oxyethylamino] 2-oxypropyltheophylline nicotinate) using the same experimental procedure. The following hypothesis was proposed to explain (a) the nonactivation and b)

the decrease of euglobulin fibrinolytic activity after repeated injection the spontaneous blood fibrinolytic activity as measured in the euglobulin fraction would have at least two origins. One component, of limited amount would be of the same origin as the activity arising after nicotinate administration. At the stage of non-activation this origin would have become nonavailable for production of the spontaneous blood fibrinolytic activity which was actually observed to be reduced. This hypothesis can also be applied to explain the observations after repeated intravenous injections of 500 mg 3-PAA indeed it is remarkable that the euglobulin fibrinolytic activity decreases to approximately the same level, whether nicotinate or 3-PAA is repeatedly injected, suggesting that both drugs make the same origin non available. After repeated injection of 150 mg 3-PAA, the first administration of which produces only moderate increase of euglobulin fibrinolytic activity this origin would still be available for production of spontaneous activity only

No modification of the Thrombotest values, the prothrombin times, the P and P-assays, factor V assays, Stypven times and thrombin times was observed after the first intravenous injection of 150 mg 3-PAA. A slight decrease of the fibrinogen level was observed after the first intravenous injection of 150 mg 3-PAA, but not after injection of 500 mg 3-PAA in the same way The fibrinogen level before and after the fifth injection of 500 mg 3-PAA was significantly increased when compared to the pre injection value. That this finding could be related to the simultaneously observed decrease of spontaneous euglobulin fibrinolytic activity is purely conjectural. No significant variation of the plasminogen level was observed after a first or after repeated intravenous injections of either 150 mg 3-PAA or 500 mg 3-PAA. INTROZZI *et al* (9) and GIBELLI *et al* (8) found a significant increase of plasminogen after one administration of 3-PAA it is however possible that the observed increase of euglobulin fibrinolytic activity interfered in their test system. GIBELLI *et al.* (8) also observed that repeated intramuscular injection of 3-PAA daily during 16 consecutive days provoked a gradual increase of plasminogen level over the sixteen day experimental period this is not necessarily in contradiction with our findings, as our experiments only lasted 16 h

A significant fall of the total leucocyte count was observed after the first injection of either 150 mg o 500 mg 3-PAA intravenously this decrease was due to a fall of the number of neutrophile

granulocytes, whereas the number of lymphocytes and monocytes remained unchanged.

A fifth injection of 150 or 500 mg 3-PAA did not modify the total leucocyte count or the leucocyte population. It is tempting to speculate that the activation of fibrinolysis by 3-PAA is in some way linked to the effect of this drug on the peripheral leucocyte count. A similar variation of the white blood cell population was also observed after intravenous injection of 3-(methyl-oxyethyl-amino) 2-oxypropyltheophylline nicotinate (3).

Summary

The modification of whole blood, plasma and euglobulin fibrinolytic activity of various clotting factors, of the plasma plasminogen level and of the leucocyte population are studied following repeated intravenous administration of either 150 mg or 500 mg 3-pyridil-acetic acid.

Résumé

Les modifications de l'activité fibrinolytique de sang complet, de plasma et d'euglobuline, ainsi que de différents facteurs de la coagulation, du taux de plasminogène plasmatique et des leucocytes ont été étudiées après l'administration intraveineuse répétée soit de 150 mg soit de 50 mg d'acide 3-pyridil-acétique.

Zusammenfassung

Nach wiederholter intravenöser Zufuhr von 150 bzw. 500 mg 3-Pyridyl-Essigsäure wurden die Veränderungen der fibrinolytischen Aktivität von Vollblut, Plasma und Euglobulin, sowie der verschiedenen Gerinnungsfaktoren, des Plasminogengehaltes im Plasma und des weissen Blutbildes untersucht.

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Parahaemophilia: The Report of One Family from Japan

M. Kagami and H. Morita

In 1943 Quick (19) observed that one-stage prothrombin time of oxalated plasma was stored in the refrigerator became progressively prolonged which was able to be corrected by adding small amounts of fresh plasma, in which the prothrombin itself was greatly depressed.

A few years later the clinical significance of this new factor became apparent from an exhaustive investigation by Owren (17) who described a female patient with bleeding tendency due to a deficiency of this factor which he originally termed factor V and later renamed proaccelerin. This deficiency condition was designated as parahaemophilia by him.

Since then about thirty additional families (3, 4, 7, 10, 21) have been reported from various parts of the world.

This communications presents the results of investigations on a Japanese male with a coagulation defect indistinguishable from that of Owren's parahaemophilia.

Case History

B. O., 30 year-old Japanese male, was referred to us on May 8, 1938, for the investigation of bleeding tendency, time when he was during the asymptomatic stage.

Haemorrhagic diathesis was first recognized by his mother who noticed subcutaneous ecchymoses appearing in the child about six months of age. Thereafter the patient experienced many bleeding episodes: easy bruising, epistaxis, severe and persistent bleeding following cuts, and dental extractions. At the age of 13, persistent bleeding following tonsillectomy continued for over 10 days, and he was once in moribund condition due to large blood loss. He developed haemarthrosis of the right knee following traffic accident when he was 25 years old. Since then, haemarthrosis of the same joint recurred twice resulting from slight trauma. Four days prior to his

admission, much haematemesis with epigastralgia occurred, from which moderate pallor of the face developed.

Family history His parents are first cousins. According to the patient statement, no incidence of excessive bleeding had been noted for three generations, except one of *his sisters, who has repeated bleeding episodes ery similar to his (fig 1) The patient and* his wife are also first cousins. There are tw children (one male and one female) with haemorrhagic episodes.

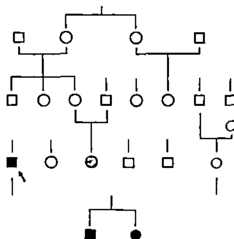


Fig 1 Family pedigree of the patient Arrow indicates propositus. Hatched Clinically affected, not tested.

Physical Examinations

Physical examination was not remarkable except for moderate anemia. There was no ecchymosis nor external bleeding The heart and lungs were normal. Abdomen was almostly negative. The liver edge was palpable under right costal margin The spleen was not palpable or enlarged to percussion. There was no adenopathy No residual deformities or functional disturbances of the right knee were evident on both physical and X ray examinations.

Examinations of the blood revealed a red-cell count of 3 080 000 haemoglobin 66 /o, and a white-cell count of 10 800 with 65.5% neutrophiles, 2 0° eosinophiles, 0.5% basophiles, 7.5 / monocytes and 24.5 % lymphocytes. The reticulocytes numbered 66°/oo and the blood platelets 422 000 per mm³ (14)

Methods

The bleeding time was performed according to Duke. I our laboratory bleeding time over 5 minutes is considered pathological.

Tourniquet test (Rumpel-Leede) was performed with the cuff of sphygmomanometer and during 5 minutes positive pressure was maintained at 10 mm under the systolic blood pressure

Whole blood coagulation time was determined according to LEE AND WHITE (11). Normal values 7 12 minutes (glass tube), 20–30 minutes (silicone coated glass tube).

Platelets were counted by the indirect method of MORIT AND YOSHIMURA (14). Normal values 325000 ± 119000 per mm³

Recalcified plasma clotting time was estimated by Howell method.

One-stage prothrombin time was measured with Quick's method and the method of ROSENFIELD AND TUFT (20)

Residual prothrombin concentration in serum was measured one hour after coagulation at 37 °C ccording to ROSENFIELD AND TUFT one-stage procedure.

Thromboplastin generation test was performed with a modification of the original method as described by BIGGS AND DOUGLAS (1 8)

All tests were carried out in oxalat system. Sera for reagent were separated after 24 hours incubation at 37 °C. Sera were diluted at least half an hour prior the tests.

Quantitati estimations of prothrombin, labile factor factor VII antihaemophilic factor were performed according to WARE-SEEGER two-stage method (23) WOLF method (24) KOLLER's method (9) and PITNEY's method (18) respectively

The plasma thromboplastin component (P T C.) activity of plasma was tested by determining its capacity to correct the abnormally long clotting time of plasma known to be deficient in P T C.

The investigation of circulating anticoagulant was performed by the determination of recalcified clotting time after addition of increasing amounts of the patient plasma to normal plasma.

Clot-retraction was measured according to MACFARLANE (13)

Plasma fibrinogen concentration was estimated by Micro-Kjeldahl method.

Platelet proaccelerin-like activity was measured by method of HJORT *et al.* (6) and modified WARE-SEEGER's method (25)

Results

1 Nature of the coagulation defect in the patient

The results of the laboratory tests on B O are summarized in Table I The bleeding time, tourniquet test, clot retraction clot lysis and fibrinogen assayed normally

The clotting time of whole blood, however was moderately prolonged in both glass and silicone coated tubes, as was recalcified clotting time. This abnormality was associated with prolongation of the one-stage prothrombin time By the modified procedure (ROSENFIELD AND TUFT) in which the test plasma is diluted with normal barium sulfate adsorbed plasma the defect measured by the customary procedure was completely corrected. Residual prothrombin in serum one hour after coagulation, measured by modified one-stage procedure was abnormally high

Thromboplastin generation test was abnormal when the reaction mixture consisted of normal platelets, normal serum

Table I
Haematologic studies in the patient.

	1958 May 11	1958 May 16	1958 Oct. 10	19
1 Routine Examinations				
Bleeding time, min (Duke)	3½		4	
Rumpel-Leede Test	negativ		negative	
Platelet Count, $\times 10^4$ (Morita)	40.2		44 7	
Whole blood clotting time				
in glass tube, min	17	22½	19½	
in silicon coated tube, min			over 180	
Recalcified plasma clotting time, second	262 (148)		337 (156)	34
2. Plasma-serum Examinations				
One-stage prothrombin time, sec				
method of Quick	22.4 (11.0)	22.6 (11 6)	19.2 (10.6)	21.
method of Rosenfield and Tuft	12.2 (12.4)		12.0 (11 8)	13.
Two-stage prothrombin concent., %		105	103	
Labile factor activity % (Wolf)	5 >	ca. 5	5 >	
Factor VII activity (Koller)		100		
A. H. F activity % (Pitney)		116	102	
P T C. activity		normal	normal	
Residual prothrombin in serum,	55		45	
Thrombin time, second		34 (38)	31 (29)	2
Circulating anticoagulant	negative		negativ	n
Plasma clot retraction, %	85		80	
Plasma fibrinogen, mg/dl			347	
Plasma clot lysis, 24 hrs, 37 °C	negativ		negativ	n
Platelet factor 1	reduced	ca. 5%	reduced	
Platelet factor 3	normal		normal	

1) Modified Ware-Seegers two-stage method.
2) Parenthesized passages indicate the control values.

barium sulfate adsorbed plasma of the patient and calcium solution (Table II)

When a mixture of equal amounts of patient's and haemophiliac's barium sulfate adsorbed plasma was us reagent in this test yielded a complete correction of the (Fig 2)

It could be concluded from different mixing experime the prolonged one-stage prothrombin time of the patient's could be corrected respectively by normal plasma, barium adsorbed normal plasma and haemophilic plasma (Table I

To exclude the possible existence of circulating antico various amounts of plasma from a normal person with a re clotting time of 148 sec were mixed with the patient's plasm his plasma recalcified clotting time was 372 seconds. As sl Table IV only 20% of normal plasma was added to the

Table II

Thromboplastin generation test.

Thromboplastin generating mixture			Time in minutes that thromboplastin generating mixture was incubated before added to substrate-plasma					
Source of platelets	Source of adsorbed plasma	Source of serum	1	2	3	4	5	6
			Clotting time in seconds of plasma to which thromboplastin generating mixture and $CaCl_2$ were added					
Normal	Normal	Normal	42.2	16.8	11.4	9.8	10.0	10.2
Normal	Normal	B. O.	39.0	17.6	10.4	9.6	9.6	9.8
Normal	B. O.	Normal	49.4	28.2	22.4	19.8	20.2	20.0
B. O	Normal	Normal	40.6	17.2	10.6	10.2	9.6	9.6
B. O	B. O	Normal	54	30.8	22.4	22.8	24.4	26.2

Table III

Correction of prothrombin time by the addition of various plasma samples.

	Mixing ratio						
Normal plasma	10			2.5			
Hemophilic plasma					2.5		
Stored norm. plasma						2.5	
BaSO adsorbed plasma		10					2.5
Patient plasma			10	7.5	7.5	7.5	7.5
Prothrombin time in seconds	11.0	360	22.4	11.4	11.6	28.6	11.2

Table IV

Correction of recalcified clotting time by the addition of normal plasma.

	Mixing ratio				
Normal plasma	10	8	5	2	0
Patient's plasma	0	2	5	8	10
Recalcified clotting time in seconds	148	115	144	188	322

plasma, the recalcified clotting time was in the normal range and thus the presence of a circulating anticoagulant was excluded

Finally quantitative estimation of the labile factor activity in the patient's plasma showed a marked reduction (5% or less of normal value) In contrast, the antihaemophilic factor (factor VIII activity was completely normal, as were the P T C. (factor IX and factor VII activities Hartert's thrombelastogram of the whole blood revealed the increased r and k value with almost normal maximum amplitude (ma value) (Fig 3)

2 Platelet factor 1 (proaccelerin-like activity) of the patient's platelets

As described before the thromboplastin generation test resulted abnormal when adsorbed plasma obtained from the patient

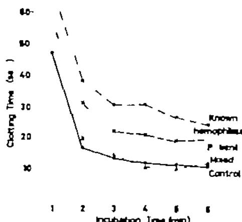


Fig 2. The correction of the clotting defect by mixing the patient' plasma and know hemophilic plasma on the thromboplastin generation test.

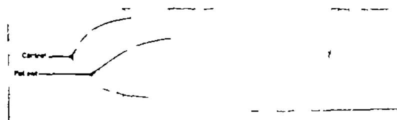


Fig 3. Thrombobelastogram using patient' whole blood. The -value 35, k-value 17 ma-value 50 and mE value 100. Normal range 15–23, k 7 13 ma 50–60, mE 100–150.

was used in reaction mixture. It was observed, as shown in Table II that the abnormality was exaggerated when the patient s platelets were used instead of normal platelets together with patient s ad sorbed plasma in the reaction mixture This observation suggests that the patient's platelets are deficient in the proaccelerin-like activity since the thromboplastic factor (platelet factor 3) activity seemed to be normal if determined by the thromboplastin generation test using normal serum and normal adsorbed plasma

The platelet factor 1 activity (as proaccelerin-like activity) was thus determined. The activity was markedly reduced (Table I) Furthermore, the fact that the defect in platelet factor 1 was correctable by exposing the abnormal platelets to normal plasma was reconfirmed (Table V)

Table I
Studies on platelet factor I

Reaction mixture	by the addition of	Clotting time (seconds)
	0.2 ml normal platelet suspension (N P S.)	41
0.2 ml factor V free plasma	0.2 ml heated—53 °C, 10 min—N. P S.	47
0.2 ml thromboplastin	0.2 ml trypsin treated N. P S.	62.
0.2 ml M/40 $CaCl_2$ solution	0.2 ml trypsin treated, resuspended in normal plasma, N. P S.	38.
	0.2 ml patient platelet suspension in saline	48.
	0.2 ml saline as control	56.

Table II
Coagulation studies of the family members (March 5, 1962).

	Wife	Son	Daughter	Control
Recalcified plasma clotting time sec	142	318	362	154
One-stage Prothrombin time (Quick) sec	12 4	13.2	15 6	12.4
Residual Prothrombi in serum, 1 h, %	10	35	32	(<15)
Factor V activity (Wolf) %	98	23	17	(80—120)
Factor VIII activity (Pitney) %	110	116	95	(60-180)
Circulating coagulant	neg	neg	neg	
Plasma clot retraction (Budtz-Olsen) %	92	88	90	(>77)

Values in parenthesis indicate the averaged normal value.

3 *Hemostatic studies on the family members*

As shown in Figure 1 the propositus patient has two children, a son and a daughter Both of them have had several hemorrhagic episodes characterized by nasal bleeding and subcutaneous ecchymoses.

The results of routine coagulation tests performed on the *patient s wife son and daughter were summarized in Table VI*

Prolonged one-stage prothrombin time and decreased factor V activity were recognized on both children but not on wife.

Discussion

The haemorrhagic tendency of the patient described at first appeared with subcutaneous ecchymoses when he was about

6 months old. Subsequently he had repeated haemorrhagic episodes including persistent bleeding following cuts, dental extractions and tonsillectomy.

The laboratory tests showed a defective coagulation mechanism in the patient's blood. One-stage prothrombin time of the patient's undiluted plasma was moderately prolonged, but it became normal after the addition of normal barium sulfate adsorbed plasma. Thromboplastin generation test was abnormal when the reaction mixture consisted of normal platelets, normal serum, barium sulfate adsorbed plasma obtained from the patient and calcium chloride. Furthermore, when a mixture of equal parts of patient's and known haemophilic adsorbed plasma was used instead of patient's adsorbed plasma alone, resulted in a complete correction of defective thromboplastin formation. These observations suggest that the substance deficient in the patient's plasma must be different from factor VIII (antihaemophilic factor). This was confirmed by the estimation of the labile factor activity in the patient's plasma, which revealed 5 % of normal or less than that. These results have led the authors to conclude that this patient had parahaemophilia, the very first case ever reported in Japan.

Parahaemophilia occurs in both sexes, but the exact mode of its inheritance (2, 5, 16) is not clear. In the present case, the parents of the patient are first cousins, and one of his sisters has a haemorrhagic tendency very similar to that of the patient. Furthermore, his two children have haemorrhagic episodes and their factor V level in the blood was proved to be very low.

Seven cases of simultaneous occurrence of the deficiencies of antihaemophilic factor (factor VIII) and labile factor (factor V) have been reported in the literature (7, 15, 21, 26).

In our cases, this possibility was excluded, since i) the known haemophiliac's plasma was capable of correcting the defective *coagulation mechanism of the patient's blood and ii) the anti*haemophilic factor (factor VIII) activity of the patient's blood was completely normal.

Ware and his collaborators (22) have demonstrated the presence of proaccelerin-like activity in normal platelets, and Lewis and Ferguson (12), as well as Hjort *et al.* (6) have reported that the parahaemophilic platelets are deficient in proaccelerin like (platelet factor 1) activity. The defect as determined by the thromboplastin

generation test was exaggerated in this case by the use of patient's platelets and barium sulfate adsorbed plasma in the reaction mixture. This observation suggests that the parahaemophilic platelets were deficient in factor 1 activity and that the platelet factor 1 in normal platelets can substitute the plasma factor V in the thromboplastin generation test at least in part (8).

Furthermore, after a series of tests the authors believe that the platelet factor 1 and factor V in the plasma are closely related and plateled factor 1 in blood platelets is perhaps resulted from the adsorption of plasma factor V from plasma.

Summary

A family of parahaemophilia is described. Propositus is 30 year-old (in 1958) Japanese male with moderately severe haemorrhagic tendency since the age of about six months. The defect in the patient clotting mechanism was attributed to deficiency of factor V (proaccelerin) in his plasma. Two children, one son and one daughter of the patient also have had bleeding manifestation very simila to his. The results of the haemostatic examinations indicated the moderate deficiency of factor V in their blood. That the parahaemophilic platelets are deficient in the platelet factor 1 activity was confirmed.

This is the first case report of parahaemophilia in Japan.

Résumé

Une famille atteinte de parahémophilie est décrit. Le propositus est un japonais âgé de 30 ans (en 1958) ayant une tendence modérée aux hémorragies depuis l'âge d à peu près 6 mois. La défectuosité dans le mécanisme de coagulation sanguine du malade fut attribuée à un manque en facteur V (proaccélérine) dans le plasma. Deux enfants, un fils et une fille du malade avaient également des manifestations hémorragiques très semblables aux siennes. Les résultats de l'examen de l'hémostase révélèren un manque modéré en facteur V dans le sang. Le manque en facteur 1 des thrombocytes parahémophiles a été confirmé. Il s'agit du premier rapport de parahémophilie au Japon.

Zusammenfassung

Es wird eine Familie mit Parahämophilie beschrieben. Der Propositus ist ein 30 Jahre alter Japaner (1958) mit einer mittelschweren hämorrhagischen Diathese seit dem Alter von ca. 6 Monaten. Die Gerinnungsstörung beruht auf einem Mangel an Faktor V (Proaccelerin) im Plasma. Ein Sohn und eine Tochter wiesen sehr ähnliche Blutungserscheinungen auf wie der Patient. In ihrem Blut fand sich ein mäßiger Mangel an Faktor V. In den parahämophilen Plättchen wurde ein Mangel an Plättchenfaktor 1 nachgewiesen. Es ist dies die erste Beschreibung einer Parahämophilie in Japan.

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Ultrastructural Aspects of Platelets and Megakaryocytes in a Case of 'Primary' Thrombocythaemia

L. Bussi, G. Jean and L. Le Coultre

The concept of thrombocythaemia implies a persistent, marked increase of the platelet-count (at least 800000/mm³) with related changes in haemostasis and the onset of a thrombophilic or an haemorrhagic state, the latter being a nearly constant finding in the majority of the cases reported.

In the present terminology primary and secondary thrombocythaemia are usually referred to. The existing literature on this subject has already been surveyed by one of the authors (1). Cases which, from the clinical-haematological standpoint, can be classified as primary thrombocythaemia are definitely found (13). On the other hand the pathological features of such cases are not substantially different from those of other myeloproliferative disorders. The assumption has thus been made that 'primary thrombocythaemia should itself be regarded as one form, or possibly one phase, of a multi-faceted myeloproliferative disorder rather than as a distinct entity' (4).

From previous investigations of platelet ultrastructure in some thrombocythaemia cases (7, 10) we concluded that certain ultrastructural differences between cases of primary thrombocythaemia and cases of splenogenous thrombocythaemia were evidenced by the electron microscope, although the observed differences could not indicate whether or not the ultrastructural changes were merely the expression of the increased thrombopoietic activity.

Recently we have examined the ultrastructure of megakaryocytes and platelets from a patient affected by 'primary' thrombo-

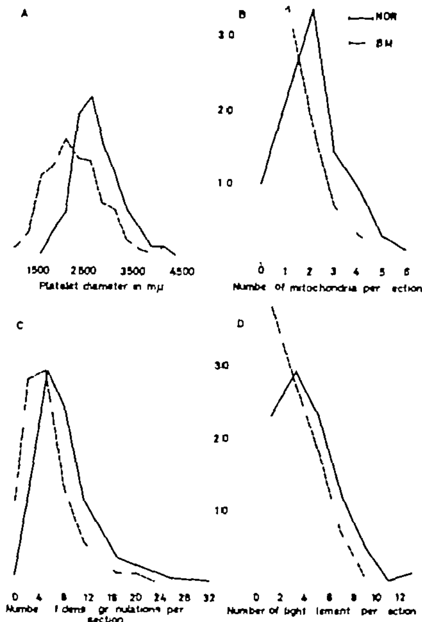


Fig 1 Electron-macroscopic findings in platelets. A) Platelet diameter in mμ. B) Number of mitochondria per section. C) Number of dense granulation per section. D) Number of ligh elements per section.

Fig 2 Polymorphic ppearance of the platelets. Platelets with plenty of cytoplasmic organelles are admixed th others (platelets 2,4) which ha almost none at all. Note oversize mitochondrion and lipid droplet in platelets 1 and 4 respectively. Areas of lower electronic density can be noted in the hyalomere of platelet 3.


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cythaemia Since we have not found in the existing literature any similar investigation, this study has been undertaken in the attempt of defining eventual ultrastructural changes characteristic of mega karyocytes and platelets in primary thrombocythaemia.

Methods

Ultrastructural investigation of plat lets and megakaryocytes was carried out at the Milan University Institute of Medical Semiology the methods hav been previously described (6—12).

Case Report

The patient, 28-year old house-wife was found slightly anaemic on medical checkup t the beginning of pregnancy (December 1962) Since then she complained of moderate fever accompanied by painful 'heaviness in the left hypochondrium. I March 1963, the patient was hospitalised because of splenic infarction. Her spleen was enlarged (4 cm below the left costal margin) The injection of 1 mg of adrenalin resulted i reduction of the spleen size The clinical course was complicated by thrombophlebitis in the right leg on the third hospital day Laboratory findings are reported in Table I.

The diagnosis of 'primary thrombocythaemia was made The patient was discharged and subjected to routine prenatal care N changes were noted in haematological checkups performed during the following months.

I September 1963, spontaneous labour resulted in stillbirth. Several infarctions could be traced in the placenta. The patient was subsequently treated with P³² the platelet-count was reduced within normal limits and clinical symptoms improved. Remission is still present.

Blood coagulation tests (clotting time determination on glass and silicone activity of prothrombin complex, prothrombin consumption, thromboplastin generation tests) hav been normal on several occasions.

Platelet enzyme determinations ha e shown higher levels tha normal (T ble II) This finding previously-observed in cases of primary and 'secondary thrombocythaemia (1) is probably rela ed to younger platelet population.

Comment In Table I our patient s and OZER s cases data are compared. The marked enlargement of the spleen was the only finding inconsistent with the diagnosis of primary thrombocythaemia However it may be worth noting that quite a few cases which had been diagnosed as primary thrombocythaemia in the past were accompanied by a marked enlargement of the spleen (4) On the other hand no case appears to ever have been reported of

Fig 3 A granulomere consisting only of light lements 18900.

Fig 4 Platelet 1 residues of platelet demarcating membranes. Platelet 2 bull' ey shaped dense granulation (arrow Platelet 3 organelles of a Golgi origin (arrow) 17500.


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Table I

Comparison between Ozer's data and our patient findings.

Conditions for diagnosis of 'primary' thrombocythaemia according to Ozer	Our patient
Thrombosis and/or haemorrhage	Thrombosis
Slight enlargement of the spleen	marked enlargement
Erythrocyte count under 6 millions	4,500 000
Haemoglobin below 18 g*	10.5 g*
Haematocrite value under 54	32
Leukocyte count under 20000	10000
Platelet count in excess of 800000	1100000
Hyperplasia of all bone marrow series	present
Megakaryocytosis	present
No leukaemic infiltrates	?

Table II

Platelet enzyme values.

	Normal	Our Patient
Aldolase	40 U	70 U
Lactodehydrogenase	63 U	170 U
G. O. Transaminase	3.5 U	6 U
G. P. Transaminase	1 U	1.9 U
Acid phosphatase	620 U	1850 U
Alkaline phosphatase	15 U	73 U

a congestive enlargement of the spleen (which was our patient's condition, in view of the considerable reduction of her spleen induced by adrenalin) associated with secondary thrombocythaemia. Generally splenomegaly and secondary thrombocythaemia are found in Hodgkin's disease, in sarcoidosis, in certain cases of spherocytic icterus or in primary fibrosis of the spleen. A congestive splenomegaly is usually associated with thrombocytopenia.

The most likely hypothesis in this case appears to be that of a primary thrombocythaemia with accompanying thrombosis of the splenic vein

Electron Microscope Findings

Platelets

The results of our electron-microscope investigation of patient's platelets are reported in Table III and Figure 1

Fig. 5. Platelets with rod-shaped dense granulations. 14000.

Fig. 6. Giant dense granulation 22750.


1μ
5
1μ
6

Table III
A comparison between platelet changes in primary thrombocythaemia and those observed in our patient.

Ultrastructural features	Primary thrombocythaemia	Our patient
Size	Microcytosis	Microcytosis
Hyalomere structure	Frequently irregular	Irregular in small percentage of sections
Glycogen	Reduced	Reduced
Steatosis	Never observed	Present
Mitochondria	Fewer	Fewer
Dense granulations	Normal or reduced number some of them non-typical	Fewer some of them non-typical
Light elements	Normal or fewer	Fewer

Size The average platelet diameter is below normal (2000 instead of 2500 mu)

Hyalomere Areas of lower electronic density (Fig 2) are shown in the hyalomere of a small percentage of platelets. Such areas are similar to those reported in some cases of primary thrombocythaemia (8 10)

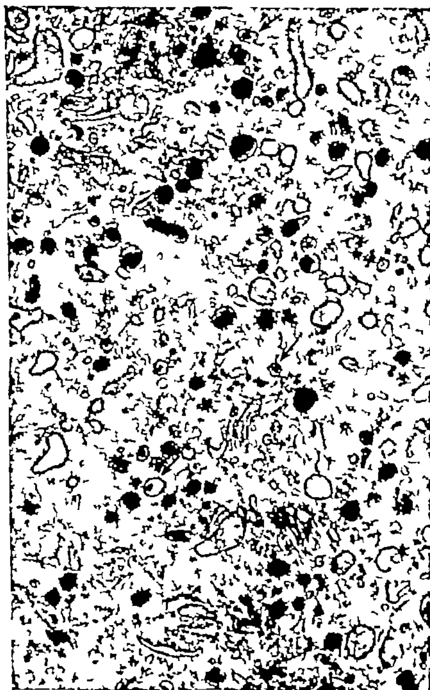
Mitochondria The number of mitochondria appears to be slightly below normal (1.3 per section instead of a normal 2-3) Particularly large mitochondria (Fig 2) were noted in 2 6 / of the sections examined.

Dense granulations The number of dense granulations, too was found to be slightly below the norm (5 instead of 7-10 per section) the reduction resulting from the high percentage of sec tions (12 ⁄) containing no dense granulation (Figs. 2 and 3) Mor phologically abnormal dense granulations were found in 18 of the sections. They were rod-shaped (8 ⁄) (Fig 5) bull s eye shaped (8 ⁄) (Fig 4) or giant size (2 ⁄) (Fig 6)

Light elements Also the number of these elements was found to be slightly below normal. While unusually large elements of this kind were seldom noted (they are instead quite numerous in secondary thrombocythaemia (8 10]) saccules and vesicles originating from the Golgi apparatus (Fig 5) were frequently observed (26% of the sections)

Steatosis Lipid inclusions (Fig 2) were found in 2.6 / of the sections.

Fig 7 Perinuclear area of megakaryocyte with large number of bull' eye shaped dense granulations (arrows Golgi apparatus (G) is well represented. 31375



Glycogen Clusters of argentaffine granules, identified as glycogen (7 9) were noted in just 26 / of the sections instead of 40 to 50 / as in normal platelets.

Megakaryocytes

Under the electron microscope (3) a normal megakaryocyte cytoplasm appears to consist of 3 concentric, structurally different areas a *perinuclear* one with plenty of mitochondria and ribosomes and a very well developed Golgi apparatus an *intermediate area* in which platelet demarcating membranes are prevalent and a *peripheral area* with no cytoplasmatic organelles. Besides a fusion of platelet demarcating membranes, a gradual maturing of granulomere organelles can be observed in the intermediate area. Particularly worth noting appears to be the gradual reduction in the size of mitochondria and the maturing of dense granulations originating from vesicles of the Golgi apparatus through several stages compound vacuoles, bull s eye shaped granulations, and dense mature granulations (12)

Our patient s megakaryocytes were featured by a remarkable polymorphism for besides a number of cells with plenty of cytoplasmic organelles (Figs. 7 8) we noted other megakaryocytes (Figs. 9 10) containing very few mitochondria, dense granulations and vacuoles, while the proportion of platelet demarcating membranes was quite normal.

Mature platelets were moreover frequently noted in the megakaryocyte center (Fig 11) They were surrounded by a vacuole equipped with a double membrane similar to the platelet demarcating ones.

Several other ultrastructural changes were ascertained in both the perinuclear and intermediate areas. The appearance of the peripheral area of these megakaryocytes was instead normal

In the *perinuclear area*, the ergastoplasmic membrane system was particularly well developed (Fig 7) and free ribosomes, too were quite plentiful. Besides the number of compound vacuoles and of bull s eye shaped granulations (Figs. 7 8) was definitely above the norm.

Fig 8. Intermediate area of megakaryocyte. Note paucity of cytoplasmic organelles. One bull' eye shaped dense granulation can also be seen (arrow) 34500.

Fig 9. Peripheral area of megakaryocyte N dense granulation is to be seen. Note permanence of particularly large mitochondrion (M) and cytoplasmic membrane (mn) 23873.


8
9

In the *intermediate area* such gradual reduction in the number and size of mitochondria which occurs in the normal megakaryocyte (3) could not be observed. As a matter of fact, particularly large mitochondria were noticed even in the cell most eccentric areas (Figs. 10 11) Maturation of dense granulations appeared to be inadequate, the number of bull s eye shaped dense granulations being elevated also in the intermediate area of these megakaryocytes.

Conclusions

The ultrastructural features of our patient s platelets and those found by one of us in other cases of primary thrombocythaemia are reported in Table II (10) Microcytosis reduction of all granulomere organelles and of intrathrombocytic glycogen, and presence of nontypical forms in dense granulations are found in our case as well as in other cases of primary thrombocythaemia on which reports are available.

In our case, a small percentage of platelets contained lipid droplets this finding so far has been reported only in a case of secondary thrombocythaemia and fibrosis of the spleen (10 11)

Finally we should like to emphasize certain ultrastructural features (particularly large mitochondria, bull s eye shaped dense granulations, Golgi apparatus residues) which are observed in both primary and secondary thrombocythaemia (10 12) The submicroscopic changes herein described were regarded as the indication of an altered platelet maturation process (3 10 12) an assumption which our findings in this particular case have confirmed. As a matter of fact, the presence of oversized mitochondria, of bull s eye shaped dense granulations and of residues of the Golgi apparatus in peripheral blood platelets is associated with marked changes in the maturation process of cytoplasmic organelles in megakaryocytes mitochondria undergo no gradual reduction in size from the perinuclear to the peripheral area, bull s eye shaped dense granulations are plentiful in the intermediate area as well, Golgi apparatus membranes and vesicles are also found in cytoplasmic areas where a very lively platelet forming activity is in progress.

Fig 10. Quite few bull' eye dense granulations and ribosomes (r) persist on megakaryocyte periphery 28750.



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Institute of Medical Pathology University of Modena (Director: Prof. E. Storti)

Regulatory Mechanisms for Protein Synthesis in Normal Blood Cells

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Knowledge of the mechanisms regulating protein synthesis is highly relevant in understanding the general phenomena which control cell turnover and are responsible for the principle immunological reactions, both humoral and cellular.

It is well established that information for protein synthesis, i.e. the sequence with which aminoacids are inserted in the polypeptide chain is conveyed by the DNA molecule. Still undetermined are instead the processes which, at a certain stage of cell life, operate the transcription of the message into a coordinated sequence of aminoacids. It is known that the mediator between DNA and protein is RNA, but only quite recently as a result of the work of biochemists and microbiologists, some insight has been gained into the mechanisms, whereby information is transferred from DNA to the protein synthesizing structures. This result has been achieved by the identification of three different types of RNA, which are structurally metabolically and functionally quite distinct ribosomal RNA, transfer RNA and messenger RNA.

Ribosomal RNA is quantitatively the most important fraction (20). It is metabolically stable (6, 27) and its molecular size is conspicuous (18–28 S or multiple of these values) (26). Its role in the regulation of protein synthesis is still far from clear although its amount has been considered to act as limiting factor (24, 15).

Transfer RNA acts as 'adaptor' and carries each aminoacid to its proper site. Although the exact number of transfer RNAs is still uncertain (5), there appears to be as many different molecular types as the number of standard aminoacids involved in protein synthesis. Their molecular size is uniform (4 S). Each aminoacid enters the polypeptide chain not because of its structure but on account of the arrangement of the bases of the transfer RNA to which it is attached. The mechanism by which transfer RNA may play part in the regulation of protein synthesis will be discussed later in connection with the findings obtained in lymphoid cells.

Messenger RNA is the fraction which appears to be most important in th
of protein synthesis.

A considerable advance in our knowledge regarding the regulation of protein synthesis was made when JACOB AND MONOD in 1961 (11) on the basis of the experimental results obtained by different investigators, elaborated a new and comprehensive *theory on the genetic mechanisms which control protein synthesis.* They suggested that each gene or DNA cistron may act as a template for the synthesis of molecules of messenger RNA, on which the polynucleotide sequence is transcribed. These messenger RNA *molecules would then attach themselves temporarily to the* ribosomes and the complex messenger RNA ribosomes would represent the functional unit concerned with protein synthesis. According to the current concepts regarding messenger RNA, this fraction is endowed with three characteristics a base *composition* complementary to that of DNA, a variable molecular size (6 to 16 S) and a high degree of metabolic instability The existence of such an RNA fraction was demonstrated in bacteria (2) and also *in animals cells (1 25 26, 7) thus indicating that the mechanism* through which the genetic message is transmitted from DNA to ribosomes appears to be fundamentally similar

The experimental proof of the existence of a messenger RNA fraction, besides its *significance as a* fundamental biochemical acquisition, was a preliminary but important confirmation of the validity of the model put forward by JACOB AND MONOD (11) for the regulation of DNA function. According to this model, a set of cistrons, residing in contiguous areas of the DNA molecule form an *operon* by virtue of the fact that their functional activity is coordinated by a common gene, the *operator* Its function consists in activating or repressing the synthesis of messenger RNAs on the operon. The operator gene in its turn undergoes the control of a *regulator* gene, which gives rise to a specific substance, the *repressor* This product directly engages the operator gene and represses it, *thus preventing the synthesis of messengers' on the operon. The* synthesis of a particular protein would take place as a result of the interaction of the repressor with a substrate called the *inductor* This reaction would inactivate the repressor and initiate the synthesis *of specific messenger RNAs. Inhibition of protein synthesis* would be due to the interaction of the repressor with a substrate, the *corepressor* By this process the repressor would become activated and would block the synthesis of new messengers on the operon. Genetic regulation therefore appears to be principally a repressive mechanism

and according to JACOB AND MONOD the main function of the
sor would consist in preventing the *formation* of messenge
rather inhibiting its function as a template for protein synth

A great deal of the experimental work carried out in
years has confirmed the general applicability of the model pr
by JACOB and MONOD and particularly the suggestion t
action of the repressor consists in preventing the formation
senger RNA rather than its function. These studies howev
mainly concerned the biochemist and the geneticist, inves
the metabolism of microorganisms. It seems needless to po
the importance which for the haematologist might ha
demonstration that mechanisms similar to those previously d
may be involved in regulating protein synthesis in blood cel
a demonstration is at present beyond our possibilities, bu
progress has been achieved by the application of autoradio
methods.

The lack of uniformity in the haemopoietic tissue,
comprises cells with markedly different functional charact
requires that a certain metabolic feature and its relatio
particular functional activity should be studied only at the
level. This requirement is met by the use of high resolutio
radiography. The autoradiographic findings hitherto obtaine
RNA and protein metabolism in different types of blood cells
the existence of two different mechanisms for the regula
protein synthesis. However since the experimental data at
available are still limited, the interpretations which will
forward must be considered purely hypothetical and present
the primary aim of stimulating new pathways of research
might confirm or modify the conclusions expressed.

Differentiating Blood Cells and the Operon Model. A Mechanism . Repression of Messenger Formation

Some of the cell systems studied, such as the granulobla
erythroblastic series, are characterized by a process of differen
which from the primitive blast cells and through inter
stages leads to the formation of mature erythrocytes and
phil polymorphs. A process of differentiation is allied w
synthesis of specific proteins and therefore the study of th
and protein metabolism during differentiation may provid

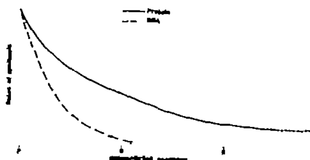


Fig. 1 Schematic representation of the variations in RNA and protein synthesis during erythroblastic differentiation. P — proerythroblasts, B — basophilic erythroblasts, Pl — polychromatic erythroblasts, O — orthochromatic erythroblasts, R — reticulocytes.

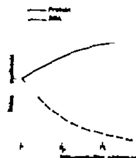


Fig. 2. Schematic representation of the variations in RNA and protein synthesis in the course of plasma cell differentiation. P — plasmoblasts, Pp — proplasmocytes, Pl — plasmocytes.

information on the mechanism by which these syntheses are regulated.

In both cell series RNA and protein synthesis is present in the precursor blast cells and decrease as maturation progresses (29 31) *However the rate of decrease is different, with the result that* in the more differentiated stages of both cell series protein formation takes place in the absence of any significant RNA synthesis (Fig 1) Furthermore about 50% of the radioactivity incorporated in both types of myeloid cells during the one hour incubation with labelled nucleosides, is linked with a highly unstable RNA, the synthesis of which ceases before that of the stable RNA fraction. The labile RNA fraction, which appears to be confined to the more immature cells of both series, was tentatively identified with messenger RNA (29 31)

As far as RNA is concerned, a sequence of events similar to that described for the granuloblastic and erythroblastic systems, also takes place in the plasma cell series (17 33) In this series, RNA synthesis decreases at the proplasmocyte stage and is almost completely absent in mature plasma cells, whereas protein synthesis steadily increases and reaches its maximum intensity only in the fully mature plasmocyte (Fig 2) Thus the increasing rate of protein synthesis distinguishes the plasma cell series from the other two differentiating cell systems and is obviously the expression of the intense globulin synthesizing and secretory activity of mature plasmocytes. In plasmoblasts and proplasmocytes from 40 to 50% of the label incorporated into RNA is unstable and once again, as for the two myeloid series, this short-lived RNA fraction was tentatively related to the presence of messenger RNA (33)

Another observation concerning a differentiating cell system, such as the erythron, has been the demonstration that haemoglobin synthesis takes place in reticulocytes in the presence of a stable RNA fraction and in conditions in which the synthesis of a DNA-dependent RNA could be excluded (14 18) Haemoglobin synthesis in reticulocytes is in fact unaffected by treatment with actinomycin D (23) It seems reasonable to assume that these stable templates are formed in the earlier maturation stages, when messenger RNA can be synthesized on the DNA primer a possibility which no longer exists in reticulocytes.

It has been further shown that even in the more immature erythroblasts protein synthesis continues unmodified for some hours after DNA inhibition by actinomycin (29) Since haemoglobin synthesis only begins in intermediate erythroblasts (28) it appears likely that also non-haemoglobin proteins are synthesized in the erythroid series on relatively stable templates. Also for these non haemoglobin proteins a certain length of time must presumably elapse between the stage when information is elaborated and that when it is utilized for the actual synthesis, on the basis of what has been observed in reticulocytes.

However the autoradiographic findings have also indicated that part of the RNA of immature cells is labile. Since metabolic instability appears to be a characteristic of messenger RNA, it seems that also in erythroid cells messenger fractions are synthesized in much the same way as in other animal cells (26, 1 25) Information is therefore elaborated in the early maturation stages.

It is possible to reconcile the apparently contradictory existence of short lived messenger fractions together with that of stable templates, on which protein synthesis takes place, if one considers that synthesis of messenger RNA occurs in the nucleus, that protein synthesis is mainly cytoplasmic and that transfer of RNA from nucleus to cytoplasm presumably requires in myeloid cells a certain interval of time. Possibly the metabolic instability of the information carrying structures is linked with the duration of their stay in the nucleus.

Elucidation of the mechanisms whereby RNA is transferred from the nucleus to the cytoplasm might certainly contribute to clarify the problem of the presence in erythroid cells of unstable messenger fractions and stable templates, but such a clarification does not appear to be immediately forthcoming (9)

The processes described, which give rise to highly specific proteins, such as haemoglobin and antibody γ-globulins, may represent a type of protein synthesis regulation, characteristic of differentiating cell systems. In a cellular system of this type it may *therefore be possible to visualize the following sequence of events* When an operator gene has been switched on in a stem cell, production of short-lived messenger RNA is initiated. Soon after DNA synthesis takes place and when the latter reaches the tetraploid value the cell undergoes mitotic division. The existence of a mutual relationship between DNA and messenger RNA synthesis may be supported by some experimental results obtained in bacteria.

In microorganisms it was found that, although DNA is not essential either for the induction or for the synthesis of the induced protein (3) the rate of the latter is dependent on the number of copies of the particular gene (12) It was also observed that the protein synthesizing capacity doubles as the particular gene is duplicated (16 8) *Notwithstanding such a relationship is still far* from having been demonstrated in differentiating *blood cells*, the above reported findings may provide an explanation for the observation that unstable RNA synthesis appears more intense in the presence of active DNA synthesis (29 31)

It may be that in haemic cells at a certain stage a repressor engages and closes the operator and the number of short lived messenger RNAs well as the rate of DNA synthesis gradually decrease. Consequently also protein synthesis comes to a halt. Obviously the production of specific proteins, which follows that of structural and

enzymatic proteins, also ceases but the arrest occurs later in the differentiating sequence. For mature plasmocytes this arrest must occur fairly late in their life-span.

The interpretations we have put forward fit in quite well with the general outline of the operon model and especially with the concept that *the progressive decrease in the rate of protein synthesis during differentiation depends on a gradual inhibition of messenger RNA synthesis rather than on a reduced utilization of preformed templates*.

Evidence for a Mechanism Involving Inhibition of Messenger Function

We will now present the findings obtained in blood cells which metabolically do not display a pattern such as one might expect from cells belonging to a particular stage of a differentiating cell system.

In our laboratory a detailed autoradiographic study was undertaken in circulating mononuclear cells on the rate of incorporation of cytidine, uridine and leucine and on the rate of breakdown of the two nucleosides after blocking RNA synthesis with actinomycin D (30–32). Peripheral blood lymphocytes were subdivided in four different groups according to their morphological features; a fifth group consisted of monocytes.

The morphological and metabolic characteristics of the different cell groups are summarized in Fig. 3. RNA and protein synthesis takes place, although at a different rate, in the majority of circulating mononuclear cells. In contrast with the findings obtained in erythroblasts, granuloblasts and plasmacells, the labelling pattern with ^{3}H-uridine differs considerably from that observed with ^{3}H-cytidine. With the latter, heavy labelling was found in immature lymphocytes, in which protein synthesis was minimal, whereas with ^{3}H-uridine the most intense radioactivity was seen in monocytes. In these cells a high ^{3}H-leucine incorporation was also observed.

The variations in the intensity of labelling may be due to the synthesis of RNAs with different functional significance. In support of this hypothesis are the differences in leucine incorporation and the different rate of release of the two nucleosides following incubation with actinomycin D. In cells with prolymphocytic features (B cells), which are labelled more rapidly with cytidine than with uridine, the former is incorporated both in stable and short lived

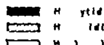
B C D E F







AVERAGE LABELLING OF RNA AND PROTEIN IN PERIPHERAL MONONUCLEAR CELLS INCUBATED FOR 1 HOUR WITH TRITIATED CYTIDINE URIDINE AND LEUCINE







AVERAGE RNA LABELLING IN PERIPHERAL MONONUCLEAR CELLS INCUBATED FOR 1 HOUR WITH TRITIATED CYTIDINE AND URIDINE AND SUBSEQUENTLY WITH ACTINOMYCIN D












H f l h ll with ctl mycl D

RNA, while uridine is only incorporated in a stable RNA. It seems likely therefore that in prolymphocytes two RNA fractions are synthesized one of these, on account of its marked instability of its presumable high uridine content* and because of the low protein synthesis with which it is associated, has been identified, in conformity with the observations of ALLFREY (1) with a messenger fraction the other RNA fraction, characterized by considerable metabolic stability may in our opinion, represent ribosomal RNA.

A third fraction, which appears metabolically unstable, but is associated with a high rate of uridine incorporation and intense protein synthesis, was observed, although in variable degree, in other types of lymphocytes and in monocytes. This fraction was considered, on a purely hypothetical basis, to represent transfer RNA. The results of experiments using phytohaemagglutinin in short-term tissue cultures of normal peripheral blood lymphocytes support the hypothesis of the existence of a transfer RNA synthesis in these types of lymphocytes. In the presence of phytohaemagglutinin (PHA) RNA and protein synthesis rapidly increases in the more common types of lymphocytes, i.e., in small and medium-sized cells (C and D) while striking morphological changes take place, leading to the emergence of large, nucleolated, pyroninophilic, primitive-looking cells (10 33) It is worth emphasizing the fact that protein synthesis already increases rapidly during the first hours of culture and that its rise proceeds in parallel with that of RNA (Fig 4) More than 50 / of the radioactivity incorporated in PHA-transformed lymphocytes, when incubated for one hour with ^{3}H uridine is linked with a metabolically unstable RNA (33)

What is the more likely interpretation of these findings? The lymphoid cells we have indicated as B cells (prolymphocytes) display metabolic features which may be compared with those of early myeloid cells, although the rate of RNA and protein synthesis is much greater in the latter Prolymphocytes are in fact charac

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I may be convenient to point out that the low incorporation of uridine, compared to that of cytidine, may be ascribed to the fact that the intracellular pool of nucleotides and therefore RNA is richer in uridine than in cytidine, the result being that labelled uridine is more diluted.

Fig 3. The morphological features of five different types of peripheral blood mononuclear cells and the pattern of labelling following incubation with tritiated cytidine uridine, leucine are shown in the upper half of the figure. I the lower half are illustrated the rates of RNA breakdown in the different types of cells after treatment with actinomycin D.

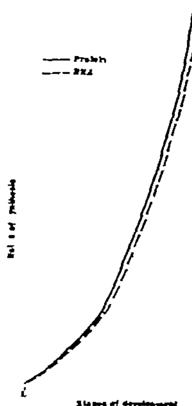


Fig. 4. Schematic representation of the variations in RNA and protein synthesis in the course of lymphocyte transformation induced by phytohaemagglutinin in vitro. L = small and medium mature lymphocytes, P = intermediate nucleolated lymphocytes with prolymphocytic features, B = primitive looking "blast" cells.

terized by a high ratio between RNA and protein synthesis and by the coexistence of a short lived RNA together with a metabolically stable fraction. In contrast, medium and large lymphocytes as well as monocytes have metabolically little in common with the more mature myeloid cells, because in the latter protein synthesis takes place in the absence of any significant RNA synthesis, whereas in the former protein production is associated with intense RNA synthesis. In our view the latter type of RNA appears to be different from the fractions predominating in the myeloid series and in plasma cells, which have been thought to represent both messenger and ribosomal RNA.

The obvious question which now arises concerns the functional significance which may be attached to the synthesis of such an RNA fraction, especially in view of the fact that great amounts of protein

are synthesized in other cell types, such as mature plasma cells, without any concomitant RNA synthesis. The question may be somewhat clarified by the events which take place when lymphocytes are cultured 'in vitro' in the presence of PHA. As previously mentioned, PHA stimulation is followed by a rapid increase in both RNA and protein synthesis. Indeed the latter increases within a few hours, long before DNA synthesis takes place and before any significant morphological changes may be observed.

It therefore seems difficult to escape the conclusion that the mechanism which regulates the synthesis of the newly-formed proteins is dissimilar from that operating in differentiating cell systems. In fact it appears unlikely that the rapid rise in protein synthesis, observed in the course of 'in vitro' lymphocytic transformation, depends upon a fairly complex set up such as would be the synthesis of messenger RNAs and their transfer to the protein synthesizing sites. Such a mechanism implies that the increase in protein synthesis should follow RNA synthesis only after a certain lag period. The existence of an interval between template formation and utilization was indeed postulated for differentiating haemic cells. It seems instead much more reasonable to assume that under these circumstances a different regulatory mechanism is required for the rapid increase in protein synthesis. This mechanism should obviously operate not by influencing the elaboration of the information carrying structures but by regulating their utilization, in other words by acting on messenger RNA function rather than on messenger RNA formation.

Is it possible to conceive the existence of such a mechanism? Recently STENT (27) has argued in favour of such a possibility. According to his hypothesis, such a mechanism might operate through the regulation of transfer RNA synthesis. Indeed only if adequate numbers of aminoacyl transfer RNAs are available, polypeptide synthesis may proceed at a sufficiently rapid rate. Owing to the probable degeneracy of the genetic code (5) some of the twenty standard aminoacids are represented by more than one triplet and are therefore cognate to more than one transfer RNA. This means that if the intracellular availability of different species of transfer RNA, cognate to the same aminoacid, is widely different, extremely variable will also be the rate of entry of the particular aminoacid in the polypeptide chain. Hence also the rate of synthesis of the polypeptide chain itself will vary considerably

A similar kind of mechanism implies that control of protein synthesis should not depend upon the formation of messenger RNA, in its turn regulated by an operator gene, but should be related to factors influencing messenger function. Stent suggests that suppression of messenger function prevents its further synthesis, indicating the possible existence of a feed-back connection between the two processes. In this particular case, in contrast to the mechanism supposedly involved in the operon model, the primary function of the repressor might consist in *inhibiting messenger function*. On the basis of the experimental results previously illustrated, we have some reason to believe that a mechanism similar to that proposed by Stent may regulate protein synthesis in lymphocytes and monocytes.

Suppression of messenger function might indeed be a common occurrence in most lymphocytes and monocytes and thus may be followed by inhibition of messenger formation. Template function may be resumed through an increased availability of transfer RNAs, the synthesis of which appears to predominate in some lymphocytes and in monocytes. PHA would possess the property of enhancing such a mechanism and, by inhibiting a repressor would stimulate both RNA and protein synthesis, which increase in parallel so that the template becomes fully activated. Following this and as a consequence of the above mentioned mechanisms, new messengers would be synthesized, their formation being no longer hampered by their defective utilization. As in the course of the differentiating process, messenger formation would be followed by DNA synthesis and cellular division. That this may be so is evidenced by the high degree of thymidine uptake and by the number of mitoses present from 48 hours onwards in PHA-cultures (21)

The scheme we have presented is purely hypothetical, but the recent findings of Cooper and Rubin (4) seem to provide some confirmation for the hypothesis put forward. These authors were able to observe in PHA-stimulated lymphocytes a sharp rise in radioactivity in the US region which would be in keeping with our interpretation that the main effect of PHA is exerted in stimulating transfer RNA synthesis.

We have given an account of the experimental observations obtained in different types of blood cells. The findings pertaining to differentiating cell systems appear to provide a further confirmation of the model proposed by Jacob and Monod while the results

derived from studies on cultures of lymphoid cells would correlate well with the scheme outlined by Stent. The latter results were of course obtained from cells cultured in a highly artificial medium. What happens to the various types of lymphoid cells in their normal environment is still a problem open to future investigations, but several considerations (13, 19, 32) would suggest that a differentiation process, involving a regulatory mechanism such as we have previously described may also take place in the lymphoid system. Whether at any particular stage these two different types of mechanism are mutually exclusive or whether they may coexist remains one of the many unsolved aspects of the fascinating and intriguing problem concerning the regulation of protein synthesis in human blood and bone marrow cells.

Summary

The experimental results hitherto obtained by different methods of approach, but especially by autoradiographic techniques, on the RNA and protein metabolism of normal differentiating blood cells and of peripheral blood mononuclear cells, are reviewed and discussed in the light of our present knowledge. The data would suggest two different mechanisms for the regulation of protein synthesis. The first, characteristic of differentiating cellular systems, would appear to follow the general principles of the operon model, involving the progressive repression of *messenger formation*. The second, pertaining to lymphoid cells and possibly monocytes, seems to conform to the scheme outlined by Stent and would consist in regulation of *messenger function* brought about by variations in the intracellular availability of transfer RNAs.

Résumé

Les résultats expérimentaux obtenus jusqu'à présent par différentes méthodes d'investigation, spécialement par l'autoradiographie, dans le domaine du métabolisme de l'ARN et des protéines de cellules sanguines se différenciant ainsi que de cellules mononucléaires du sang périphérique, sont discutés à la lumière de nos connaissances actuelles. Ils suggèrent la présence de deux mécanismes différents réglant la synthèse des protéines: le premier qui est caractéristique pour les systèmes cellulaires se différenciant semble apparemment suivre les principes généraux du modèle «operon» comportant une *inhibition progressive* de la formation de l'ARN *messager*. *Le second* a trait aux cellules lymphoïdes et peut-être aux monocytes. Il semble correspondre au modèle proposé par Stent et consisterait en une régulation de la fonction messagère réalisée par des variations de la disponibilité intracellulaire de l'ARN de transfer.

Zusammenfassung

Die Ergebnisse verschiedener Untersuchungsmethoden, insbesondere der Autoradiographie, auf dem Gebiete des RNA- und Eiweißstoffwechsels von normalen differenzierenden Blutzellen und von mononukleären Zellen des peripheren Blutes werden im Lichte unserer derzeitigen Kenntnisse erörtert. Sie sprechen für das Vorliegen

differenzierende Zellsysteme und scheint den allgemeinen Prinzipien des Operon-Modells mit progressiver Unterdrückung der Bildung von «Messenger-RNA» zu folgen. Der zweite betrifft die lymphoiden Zellen und wahrscheinlich auch die Monozyten er dürfte dem Schema von STENT entsprechen und beruht auf einer Steuerung der «Messenger»-Funktion durch Veränderungen der intrazellulären Wirksamkeit von Transfer RNA»

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Methode und Material

Methode. Die Vitamin B_{12}-Bestimmung im Serum wurde mit der biologischen Methode nach *USP 16 mit Hilfe von Lactobacillus Leichmannii (Stamm ATCC 7830)* durchgeführt (26). Der Serumaufschluß erfolgte nach ROSENTHAL und SARETT (24). An Stelle des 1%igen Acetatpuffers verwendeten wir den Standard-Acetatpuffer der Firma Merck (pH 4,62). Das Serumpuffergemisch wurde auf pH 5,3 eingestellt, da sich bei pH 5,1 die Proteine nicht vollständig abtrennten. Die Eichkurve wurde mit Cyanocobalamin (Cytobion® der Firma Merck) aufgestellt*.

Die Konzentration des Vitamin B_{12} wird durch seine wachstumsfördernde Wirkung auf den L. Leichmannii bestimmt. Die durch das Bakterienwachstum entstandene Trübung wird optisch gemessen. Sie entspricht der Menge des vorhandenen Vitamin B_{12}.

Material. Wir untersuchten insgesamt 23 gesunde Personen und 300 Patienten mit verschiedenen Erkrankungen, darunter 119 mit hämatologischen. Bei nahezu allen Patienten standen uns die Werte des peripheren Blutbildes zur Verfügung, die zum Zeitpunkt der Vitamin B_{12}-Bestimmung angefertigt wurden. Bei 143 Patienten konnten wir zusätzlich Sternalpunktionen durchführen. Die Vitamin B_{12}-Werte, vor allem die außerhalb der Norm liegenden, wurden durch Doppel- oder Mehrfach-Bestimmungen gesichert. Die bei 23 gesunden Patienten ermittelten Normwerte lagen zwischen 150 und 650 $\mu\mu$g Vitamin B_{12}/ml Serum. Sie befanden sich damit in Übereinstimmung mit den in der Literatur angegebenen Werten, die mit den gleichen oder anderen Methoden ermittelt worden waren, weshalb wir auf eine größere Kontrollgruppe verzichteten (16, 21). Wegen des großen Streubereiches des Vitamin B_{12} im Serum haben wir bewußt auf die Angabe von Mittelwerten verzichtet. Patienten, bei denen nachweisbar eine Vitamin B_{12}-Therapie vorausgegangen war, wurden eliminiert, auch wenn diese Behandlung Monate zurücklag. Ebenso haben wir Patienten, die vorher unter einer antibiotischen Therapie standen, aus dem Untersuchungsgut herausgenommen, weil durch Antibiotica im Serum das Wachstum des L. Leichmannii gehemmt wird und fälschlich niedrige Vitamin B_{12}-Werte entstehen.

Ergebnisse

Die Aufschlüsselung des Krankengutes nach Krankheitsgruppen und die Höhe der einzelnen Vitamin B_{12}-Werte ist aus Tabelle I ersichtlich. In Tabelle II werden zusätzlich zu den Vitamin B_{12}-Werten 143 Knochenmarksbefunde angegeben. Die Werte der Vitamin B_{12}-Bestimmung bei Magenresezierten und die dazu gefundenen hämatologischen Befunde werden in Tabelle III besonders berücksichtigt.

Diskussion

Wenn man die Ergebnisse überschaut, dann fällt auf, daß sich in fast allen Krankheitsgruppen Patienten mit niedrigen Vitamin B_{12}-Werten finden. Betrachtet man dabei die Gruppen, bei denen

* Wir danken der Firma Merck AG, Darmstadt, für die Überlassung der Bakterienstämme und Testlösungen.

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I. Med. Abteilung des Städtischen Krankenhauses München-Schwabing
(Chefarzt: Prof. Dr. H. Begemann)

Der Wert der Vitamin B_{12}-Bestimmung im Blutserum für die klinische und hämatologische Diagnostik*

H. Begemann, J. Rastetter, R. Wauble
und H. Ketterer

Die Bestimmung des Vitamin B_{12} im Serum hat sich bei der Aufklärung von Vitamin B_{12} Mangelzuständen als wertvoll erwiesen. So konnte für die perniziöse Anämie eine «kritische» Grenze, die bei einem Vitamin B_{12}-Serum-Spiegel von 100 $\mu\mu$g/ml lag, fest gelegt werden. War der Serum Vitamin B_{12}-Gehalt unter diese Grenze abgefallen, sollten die bekannten hämatologischen und klinischen Störungen in Erscheinung treten. Wir stellten uns die Frage, ob bei einem größeren Krankengut neben der Perniciosa ein Vitamin B_{12} Mangel festzustellen wäre und ob sich die Höhe des Vitamin B_{12}-Gehaltes im Serum mit klinischen Befunden und hämatologischen Daten korrelieren ließe. Dazu zogen wir als Kriterien den Hämoglobingehalt/100 ml Blut, den Hämoglobingehalt des Einzelerythrozyten (Hb$_E$) und den Sternalmarkbefund heran. Von Interesse erschien auch, ob dabei passagere Vitamin B_{12}-Mangelzustände zu finden seien, die durch andere Untersuchungen nicht erfaßt würden. Im Gegensatz zu Kahn (16) bei dem von 57 Patienten mit hämatologischen Erkrankungen nur ein Fall einen pathologischen Vitamin B_{12}-Gehalt aufwies, fanden wir solche Fälle in unserem Krankengut wesentlich häufiger. Außerdem war es wertvoll festzustellen, ob bei bestimmten Krankheitsbildern, ähnlich der perniziösen Anämie, charakteristische unter bzw. über der Norm liegende Vitamin B_{12}-Werte vorhanden waren, die eventuell als differential diagnostisches Kriterium dienen könnten.

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* Herrn Prof. Dr. J. Frey, Frankfurt/Main zum 60. Geburtstag.

Die Untersuchungen wurden mit Unterstützung der Deutschen Forschungsgemeinschaft durchgeführt.

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Tabelle I

Vitamin B_{12}-Gehalt im Serum bei verschiedenen Krankheiten

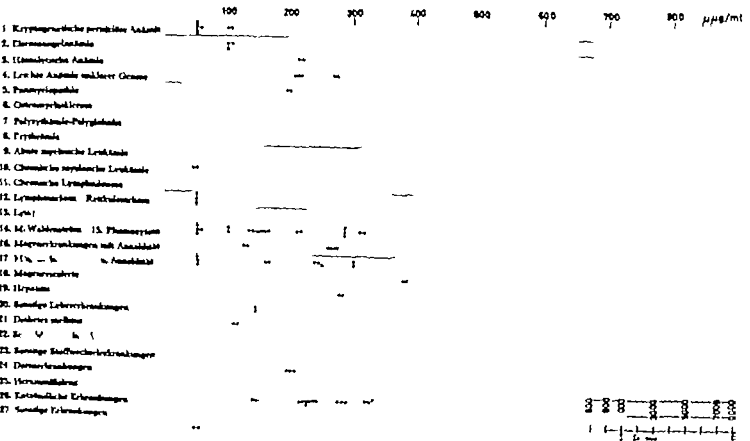


Tabelle II
Vitamin B_{12}-Gehalt im Serum, Knochenmarkbefunde.

Krankheit	Zahl der Fälle	B_{12}-Gehalt im Serum μμg			Davon Stern. Punkt.	Megaloblast. Veränderungen	
		<150	150–650	>650		vorhand.	fehlen
1. Perniziöse Anämie	19	14[3]			12	12	0
			5[3]		5	5	0
2. Eisenmangel-	7	3			3	0	3
Anämie			4		2	0	2
3. Hämolytische	6		6		5	0	5
Anämien							
4. Leichte Anämien	9	3			0		
unklarer Genese			6		1	0	1
5. Panmyelopathie	12	4			3	2	1
			7		7	1	6
				1	1	0	1
6. Osteomyelosklerose	7	2			2	0	2
			5		2	0	2
7. Polycythaemia vera	3	3			3	2	1
Polyglobulie	2		2		2	0	2
8. Erythrämie	4	1			1	1	0
			3		3	3	0
9. Akute myel.	8	1			1	0	1
Leukämie			7		6	1	5
10. Chronische myel.	27	2			2	1	1
Leukämie			6		4	0	4
				19	12	0	12
11. Chronische	13	5			4	0	4
Lymphadenose			8		7	0	7
12. Lympho- und	4		4		3	0	3
Retikulosarkom							
13. Lymphogranulo-	12	5			1	0	1
matose			7		3	0	3
14. M. Waldenström	4	3			3	3	0
			1		1	0	1
15. Plasmocytom	1		1		1	0	1
16. Magenerkr. mit	34	10[4]			6	3	3
Anacidität			22		5	0	5
				2	0	0	0
17. Magenerkr. ohne	19	3[4]			2	1	1
Anacidität (Ulcera,			15		1	0	1
Gastritis, Neoplasma)				1	0		
18. Magenresektion	25	5[3]			3	3	0
			19[4]		8	1	7
				1	1	0	1
19. Hepatitis	7		7		1	0	1
20. Sonstige Lebererkr.	9	1			0		
vorwieg. Cirrhosen			8		3	0	3
21. Diabetes mellitus	7	2			1	0	1
			5		0		
22. Schilddrüsen-	8	5			0		
erkrankungen			3		0		

Tabelle II (Fortsetzung)

Krankheit	Zahl der Fälle	B_{12}-Gehalt im Serum µµg			Davon Sternal-Punkt.	Megaloblast. Veränderungen	
		<150	150–650	>650		vorhand.	fehlen
23. Sonstige Stoffwechs. Erkr. (Haemodd., Porphyr. gen. Fam.)	5	3			1	0	1
			1		0		
				1	0		
24. Erkrank. d. Darmes	3	3			3	0	3
25. Herzinsuffizienz	12	3			0		
			9*		1	1	0
26. Entzündl. Erkrank.	9	2			1	0	1
			7		3	0	3
27. Sonstige Erkrank.	24	5			1	0	1
			18		2	0	2
				1	0		
	300	88	186	26	143	40	103

je 1 dimorphe Anämie
2 dimorphe Anämien
1 dimorphe Anämie

Patienten mit pathologischem Vitamin B_{12}-Spiegel deutlich überwiegen, so heben sich einige Krankheitsbilder besonders ab.

Erwartungsgemäß war die Verschiebung in dem pathologisch erniedrigten Bereich bei der *perniziösen Anämie* am hervorstechendsten. Doch fanden sich immerhin 5 Fälle mit normalem Serum-Vitamin B_{12}, so daß ein Vitamin B_{12}-Mangel als Ursache der megaloblastischen Veränderungen auszuscheiden ist. Am nächstgelegenen wäre ein Folsäure-Mangel zu diskutieren.

Trotz der geringen Anzahl der untersuchten Patienten mit *Polycythaemia vera* und *Polyglobulie* zeichnete sich bei diesen Kranken eine Erniedrigung des Vitamin B_{12}-Spiegels ab. Zusammenhänge zwischen den in der Literatur mitgeteilten Fällen (4, 9, 11, 27, 31) über die wechselseitigen Beziehungen zwischen Polyzythämie und megaloblastischen Anämien könnten damit erklärbar werden. Es wird angenommen, daß durch starke Zellproliferation der Erythropoese ein ständiger Mehrverbrauch an Vitamin B_{12} entsteht, aus dem schließlich ein Vitamin B_{12}-Mangel resultiert, der zur megaloblastischen Anämie führen kann. Der Beweis für einen Vitamin B_{12}-Mangel konnte bei den Fällen aus dem Schrifttum dadurch belegt werden, daß es nach genügender Vitamin B_{12}-Zufuhr wieder zur Polyzythämie kam.

Ebenfalls als Folge eines vermehrten Vitamin B_{12}-Verbrauchs durch gesteigerte Zellproliferation könnte der erniedrigte Vitamin B_{12}-Gehalt bei *Lymphadenosen* angesehen werden, der bei unseren Kranken im unteren Normbereich oder darunter lag. Gleiche Beobachtungen machte ERDMANN-OEHLECKER (10) bei 21 Lymphadenosen im Gegensatz zu MOLLIN UND ROSS (20), deren 9 Fälle ein normales Serum Vitamin B_{12} aufwiesen.

VAN DOMMELEN *et al.* (8) erklären einen Fall von megaloblastischer Anämie bei *Morbus Waldenström* (M. W.) ebenfalls durch den erhöhten Verbrauch des Vitamin B_{12} infolge der plasmazellulären Neoplasie. Bei den von uns untersuchten 4 Patienten mit M. W. waren die Vitamin B_{12}-Werte dreimal deutlich unter dem Normbereich. Diese Fälle waren auch durch megaloblastische Markveränderungen gekennzeichnet. KILLANDER (17) sah dagegen bei seinen drei Fällen mit M. W. normale Vitamin B_{12}-Werte, jedoch bei Plasmocytomen erniedrigte. Unser *Plasmocytom*-Fall hatte dagegen ein normales Serum Vitamin B_{12}.

Die Ursache der Vitamin B_{12}-Mangelzustände durch Mehrverbrauch bei den oben angeführten Krankheitsbildern ist denkbar. Berücksichtigt man aber die Befunde bei den akuten und besonders bei den chronischen myeloischen Leukämien, also Erkrankungen, bei denen die Proliferation des hämopoetischen Systems wohl am stärksten ist und daher der Vitamin B_{12}-Verbrauch am größten sein sollte, so wird man diese Meinung nicht mehr vertreten können. In Übereinstimmung mit anderen Autoren fanden wir den Vitamin B_{12}-Gehalt im Serum bei den meisten *akuten myeloischen Leukämien* im Normbereich, bei *chronischen myeloischen Leukämien* meist weit oberhalb der Norm (2, 10, 15, 16, 20, 23). Eine ausreichende Erklärung dieses Phänomens steht bis heute noch aus. Die Vermutung, daß die Höhe des Vitamin B_{12}-Gehalts von der Zahl der Leukozyten (3, 20), dem Anteil der Basophilen (15) oder dem Anteil der unreifen Blutzellen in der Peripherie (3) abhängig sei, ließ sich durch unsere Untersuchungen nicht bestätigen. Man wird eher annehmen dürfen, daß für die Vitamin B_{12}-Erhöhung grundsätzlich andere Faktoren verantwortlich sind. (Aus diesem Grund wurden chronisch myeloische Leukämien in Tabellen IV und V nicht berücksichtigt, s. u.) Wir werden diese Befunde jedoch an anderer Stelle noch ausführlich diskutieren.

Hervorzuheben sind in diesem Zusammenhang die bei der *Osteomyelosklerose* gefundenen niedrigen oder normalen Vitamin B_{12}-

Werte. Möglicherweise ergeben sich daraus wertvolle differentialdiagnostische Hinweise gegenüber der chronisch myeloischen Leukämie, wie auch weitere pathogenetische Gesichtspunkte.

Beim größeren Teil unserer Patienten mit *Schilddrüsenerkrankungen* lag der Vitamin B_{12}-Spiegel im Normbereich und darunter. Diese Befunde sind wichtig, weil in den letzten Jahren Zusammenhänge zwischen der perniziösen Anämie und Schilddrüsenerkrankungen (Hypo- bzw. Hyperthyreosen) aufgedeckt wurden. Als Ursache werden immunologische Vorgänge angenommen, da bei beiden Krankheiten zu einem großen Prozentsatz Antikörper sowohl gegen Schilddrüsengewebe als auch gegen Parietalzellen der Magenschleimhaut nachweisbar sind (7, 14, 19).

Bei den Fällen mit *Hämochromatose* war der Vitamin B_{12}-Spiegel wie bei anderen Untersuchern (18) stark erniedrigt. Megaloblastische Anämien waren dabei im Gegensatz zu den in der Literatur niedergelegten Einzelberichten nicht oder noch nicht vorhanden. Auf Grund des Vitamin B_{12}-Mangels wäre es denkbar, daß bei unseren Patienten noch mit dem Auftreten einer megaloblastischen Anämie zu rechnen ist. Andererseits sind Fälle mit Hämochromatose beschrieben worden, bei denen die megaloblastischen Veränderungen eindeutig auf einen Folsäuremangel (29) oder Mangel an Vitamin B_6 (28) bezogen werden konnten. Als Ursache eines Vitamin B_{12}-Mangels könnte man Veränderungen der Magen-Darmschleimhaut (verminderte Intrinsic-factor Bildung, gestörte Resorption) oder eine verminderte Speicherungsfähigkeit der erkrankten Leber diskutieren.

Neuere Untersuchungen haben gezeigt, daß Patienten mit *Anacidität* des Magensaftes ein deutlich höherer Prozentsatz mit Antikörpern gegen Parietalzellen gegenüber Normaciden gesehen wird. Damit ergeben sich Parallelen zur kryptogenetischen perniziösen Anämie (25). So fanden BALAZS *et al.* (1) im Serum von 78,5% ihrer Perniziosa-Kranken gegen Magenschleimhaut gerichtete Antikörper, desgleichen bei 43% der Patienten mit Achlorhydrie, aber nur bei 8,2% der Kontrollen. Prüft man nun bei den anaciden Patienten lediglich den Vitamin B_{12}-Gehalt im Serum, so überrascht bei unseren Untersuchungen die relativ hohe Zahl (30%) mit einer B_{12}-Hypovitaminose (unter 150 $\mu\mu$g/ml). Nimmt man dazu noch die Patienten, bei denen der Vitamin B_{12}-Spiegel im unteren Normbereich lag (unter 200 $\mu\mu$g/ml), so erhöht sich die Zahl sogar auf 50%. Magenkranke ohne Säureverlust wie auch

Tabelle III

Vitamin B_{12}-Gehalt im Serum und hämatologische Befunde bei Magenresezierten.

	Geschl.	Alter J.	Zeit nach der Op.	B_{12} i. S. µµg	Op.-Art	Hämatolog. Befunde Knochenmark	Blut	Bemerkungen
1.	M	61	39 Jahre	191	?	megalobl. Anämie	Hypochr Anämie	Dimorphe Anämie
2.	M	75	33 Jahre	915	?	o. B.	o. B.	
3.	M	68	23 Jahre	166	?	—	o. B.	
4.	M	52	16 Jahre	142	?	—	normochr Anämie	
5.	M	43	16 Jahre	< 100	Billroth II	megalobl. Anämie	hypochr Anämie	Dimorphe Anämie
6.	W	48	14 Jahre	< 100	Billroth II	megalobl. Anämie	hyperchr Anämie	
7	M	60	13 Jahre	437	Billroth II	o. B.	hypochr Anämie	
8.	M	62	12 Jahre	310	Billroth II	Farbst. Anämie	hypochr Anämie	
9	M	43	12 Jahre	246	Billroth II	—	normochr Anämie	
10.	M	47	10 Jahre	252	?	—	o. B.	
11	M	63	9 Jahre	301	Billroth II	—	o. B.	
12.	W	60	9 Jahre	240	?	o. B.	o. B.	
13.	M	52	5 Jahre	335	?	Farbst. Anämie	o. B.	
14	M	59	5 Jahre	324	Billroth II	—	o. B.	
15.	W	49	4 Jahre	12	Billroth II	—	o. B.	
16.	M	72	3 Jahre	< 100	subdiaph. Fundekt.	megalobl. Anämie	hyperchr Anämie	
17	M	43	2 Jahre	376	Billroth II	—	normochr Anämie	
18.	M	?	1 Jahr	214	?	—	—	
19	W	76	0 Jahre	162	Billroth II	—	normochr Anämie	
20	M	42	0 Jahre	617	Billroth II	—	normochr Anämie	
21	W	48	0 Jahre	347	Billroth II	Farbst. Anämie	normochr Anämie	
22.	W	53	0 Jahre	415	Billroth II	—	normochr Anämie	
23.	M	53	0 Jahre	252	Totale Magenres.	—	normochr Anämie	
24	W	68	0 Jahre	300	Billroth II	Farbst. Anämie	hypochr Anämie	
25.	W	72	0 Jahre	646	Billroth II	KM-veröd. Prozeß	normochr Anämie	

Magenresezierte wiesen fast ausschließlich normale Serum-Vitamin B_{12}-Verhältnisse auf. Wie aus der Tabelle III hervorgeht, war beim größten Teil der Patienten eine Magenresektion nach BILLROTH II durchgeführt worden. Über die Häufigkeit des Auftretens

von megaloblastischen Anämien nach *Magenresektion* liegen keine bindenden Zahlen vor. Man weiß aber, daß sowohl die Ausdehnung der Resektion als auch die Zeitspanne nach der Operation für den Beginn einer megaloblastischen Anämie verantwortlich zu machen ist (22). Resorptionsversuche (SCHILLING-Test) ergaben nicht nur bei totaler Magenresektion, sondern auch bei partiell Resezierten – wenn auch in geringerer Anzahl – eine der perniziösen Anämie vergleichbar gestörte Vitamin B_{12}-Aufnahme (6, 13). Im allgemeinen gilt ein Zeitraum von 3 bis 4 Jahren post operationem als kritisch, da nach dieser Zeit das Vitamin B_{12} Depot der Leber erschöpft ist. Von unseren Patienten bekamen nach dieser Zeit nur vier eine megaloblastische Anämie, zwei davon eine dimorphe Anämie (s. u.). Insgesamt überwiegen in unserem Krankengut, wie auch bei anderen Untersuchern (30), Farbstoffanämien. Möglicherweise läßt sich auch die geringe Anzahl von Vitamin B_{12}-Mangelzuständen durch die weit verbreitete Anwendung von Vitamin B_{12}-Präparaten erklären. Drei Patienten mit *Darmerkrankungen* hatten unter der Norm liegende Vitamin B_{12}-Werte. Während die verminderte Vitamin B_{12}-Resorption bei einem Patienten mit Malabsorptionssyndrom durch die Mitbeteiligung der Vitamin B_{12}-resorbierenden Anteile des Dünndarms verständlich ist, ist bei den zwei Patienten mit Colitis ulcerosa eine Deutung nicht ohne weiteres möglich. Wahrscheinlich wird die Aufnahme des Vitamins durch die gleichzeitig bestehenden Resorptionsstörungen im gesamten Magen-Darmtrakt (beschleunigte Magen-Darm Passage, entzündlichen Veränderungen im Bereich der Vitamin B_{12}-resorbierenden Darmanteile) gehemmt.

Bei Betrachtung der Vitamin B_{12} Werte der anderen Krankheitsgruppen (Nr. 2–8, 12, 13, 15, 19–21, 23, 25–27 der Tabellen I und II) ist trotz Fehlens charakteristischer Veränderungen für einzelne Erkrankungen wiederum die nicht geringe Zahl der Patienten bemerkenswert, die einen pathologisch erniedrigten Vitamin B_{12}-Gehalt im Serum aufweisen. Die auffallend seltene Manifestation megaloblastischer Anämien läßt darauf schließen, daß es sich mit Wahrscheinlichkeit um passagere Zustände handelt. Diese Annahme wurde durch spätere Kontrollen bestätigt, bei denen ohne therapeutische Vitamin B_{12}-Zufuhr eine Normalisierung der Werte festzustellen war. Sicher spielen bei der Entstehung des B_{12}-Defizits mehrere Faktoren eine Rolle. So könnte z. B. im Verlauf von kardialen Dekompensationszuständen die durch die Stauungs-

Tabelle IV

Beziehungen von B_{12}-Gehalt im Serum, Hämoglobin (Hb g/100 ml) und Einzelery (Hb_E) (mit Ausnahme von chron. myel. Leukämien und Erythrämien)

B_{12}-Gehalt im Serum µµg	Hämoglobin g/100 ml arithm. Mittelw.	Streuung (s)	Hämoglobin d. Einzelery. (Hb_E) arithm. Mittelwert	Streuung (s)
0–100	12,8	3,8	33,7	4,8
100–150	12,7	3 4	30,9	4 4
150–250	12,4	3,1	32,2	3,9
250–350	12,9	3,0	31 4	2,8
350–450	11,6	3,4	32,6	2,6
450–650	12,8	3,1	31,6	2,5
über 650	13,6	3,0	32,8	1,2

erscheinungen gestörte intestinale Resorption, bei degenerativen Lebererkrankungen eine Änderung der Depotfunktion von Bedeutung sein. Die therapeutischen Konsequenzen, die sich aus einem erniedrigten Vitamin B_{12}-Spiegel im Serum ergeben, sollen zunächst dahingestellt bleiben.

Bei der Besprechung der Krankheiten war bereits die oft erhebliche Differenz der Vitamin B_{12}-Werte innerhalb der Gruppen aufgefallen. Wir prüften nun, ob der Höhe des Vitamin B_{12}-Spiegels ein spezifisch hämatologisches Untersuchungsergebnis zugeordnet werden kann. Teilt man, wie das in Tabelle IV durchgeführt ist, den Gehalt an Serum-Vitamin B_{12} in bestimmte Größen ein und stellt diesen die dazu ermittelnden Hb-Werte (Mittelwerte) gegenüber so ergeben sich keine festen Korrelationen. Auffallend erscheint besonders, daß in der Gruppe von 0 bis 100 $\mu\mu g$/ml also bei erheblichen B_{12}-Hypovitaminosen, keine stärkere Anämie nachweisbar war Dieser Befund könnte wieder auf latente bzw passagere Vitamin B_{12}-Mangelzustände hinweisen. Allerdings würde sich das Bild etwas ändern und eine Anämie deutlicher werden, wenn unsere Patienten mit Polycythaemia vera, die bei hohem Hb einen deutlichen Vitamin B_{12}-Mangel im Serum aufwiesen, in dieser Gruppe unberücksichtigt blieben.

Setzt man nun den Hb-Gehalt des Einzelerythrozyten (Hb_E) in Beziehung zu den angeführten Größenbereichen des Serum-Vitamin B_{12}, so lassen sich auch hier wiederum keine signifikanten Abweichungen unter den einzelnen Gruppen nachweisen. Dieser Befund ist deshalb erstaunlich, da beim Vitamin B_{12}-Mangel das Auftreten einer Hyperchromie zu erwarten wäre. Kommt es doch beim Vitamin B_{12}-Defizit im wesentlichen zu einer erheblichen

Störung in der Zellbildung, während die Abweichungen in der Hämsynthese weniger in Erscheinung treten. Die Folge ist, daß die Erythrozyten stärker als normal mit Hb gefüllt werden, dementsprechend das Hb_E über $34\gamma\gamma$ ansteigt. Aus dem Fehlen einer Hyperchromie bei einer heterogenen Gruppe von Patienten, die lediglich durch den Vitamin B_{12}-Mangel im Serum gekennzeichnet ist, läßt sich folgern, daß ein pathologisch erniedrigtes Vitamin B_{12} nicht mit einer Hyperchromie einherzugehen braucht. Noch eindeutiger wird dieser Befund, wenn man die bei den einzelnen Patienten ermittelten Werte des Hb_E und des Serum Vitamin B_{12} betrachtet. Fanden wir doch auch Hyperchromie bei normalem oder erhöhtem Vitamin B_{12}-Spiegel wie auch hypochrome Anämien beim Vitamin B_{12}-Mangel, selbst wenn man die eindeutig definierten dimorphen Anämien ausklammert.

In diesem Zusammenhang soll auch auf die verhältnismäßig große Zahl der Patienten mit *dimorphen Anämien* hingewiesen werden, wie sie auch unter anderem auch von BEL (5) beobachtet wurden. Man versteht darunter hypochrome Anämien in Kombination mit megaloblastischem Knochenmark. In unserem Krankengut fanden sich immerhin 8 Fälle, bei denen dieser Befund zutraf. Erfahrungsgemäß sprechen diese Patienten therapeutisch nur auf eine Kombination von Eisen und Vitamin B_{12} an.

Als Kriterium eines Vitamin B_{12}-Mangels gilt u. a. das Auftreten von Megaloblasten im Knochenmark. Daher schien es uns wichtig zu prüfen, ob sich zwischen dem Vitamin B_{12}-Gehalt im Serum und dem Knochenmarkbefund Beziehungen herstellen ließen. Von 86 untersuchten Patienten ohne megaloblastische Veränderungen im Knochenmark wiesen 23 (=27%) einen niedrigen Vitamin B_{12}-Gehalt im Serum auf. Aber auch von 35 Patienten mit Megaloblasten hatten 10 Fälle einen normalen Vitamin B_{12}-Spiegel. Der erste Befund könnte damit erklärt werden, daß nicht nur der Vitamin B_{12} Mangel als Ursache einer Megaloblastose angesprochen werden kann. Am naheliegendsten müßte ein Folsäuremangel berücksichtigt werden, womit sich die Notwendigkeit von Folsäurebestimmungen im Serum ergibt. Schwieriger zu interpretieren ist die fehlende Megaloblastose beim Vitamin B_{12} Mangel.

Folgende Möglichkeiten müßten zur Diskussion gestellt werden.

1. *Der Vitamin B_{12}-Mangel muß eine gewisse Zeit bestanden haben,* um zur morphologisch-manifesten Störungen im Nucleinsäurenstoffwechsel zu führen. Für diese Annahme könnten die Befunde bei Gastrektomierten sprechen, bei denen es durch die

Tabelle V

Beziehungen zwischen Knochenmark-Befunden und B_{12}-Gehalt im Serum (ausgenommen Erythrämien und chron. myel. Leukämien)

Knochenmark	Anzahl der Fälle	B_{12}-Gehalt im Serum (µµg) unter 150	150–650 (Normalwert)	über 650
Ohne megaloblast. Veränderungen	86	23 (27%)	61 (71%)	2 (—2%)
Mit megaloblast. Veränderungen	35	25 (72%)	10 (28%)	0 (0%)

fehlende Vitamin B_{12}-Resorption zu einer Erschöpfung der Vitamin B_{12}-Depots kommt und schließlich eine Megaloblastose auftritt (22, 30). Dagegen spricht aber das Verhalten von Vegetariern, bei denen trotz niedrigem Vitamin B_{12}-Spiegel niemals Megaloblasten beobachtet werden (12)

2. Zum Vitamin B_{12}-Mangel müssen noch andere zusätzliche Faktoren hinzukommen, um eine Megaloblastose zu erzeugen.

3. Bestimmte Stoffwechselfunktionen des Vitamin B_{12} können durch Folsäure übernommen werden. Es wird vermutet, daß die in normaler Menge vorhandene Folsäure eine Mobilisierung des quantitativ reduzierten Vitamin B_{12} aus anderen Stoffwechselsystemen bewirkt, wodurch für die Hämopoese noch ausreichende Mengen Vitamin B_{12} zur Verfügung stehen. Beim Folsäuremangel kann diese Funktion durch das Vitamin B_{12} übernommen werden (32). Diese Annahme wird gestützt durch die Beobachtung,daß schon geringste Mengen Vitamin B_{12} (0,1 µg) beim Perniziosakranken eine Besserung des Blutbildes herbeiführen können (33)

Die *Erythrämien*, die in unserem Krankengut, ausgenommen ein Fall, normale Vitamin B_{12}-Spiegel aufwiesen aber alle schwerste megaloblastische Knochenmarksveränderungen zeigten, sind hier bei (Tabellen IV und V) nicht berücksichtigt. Die Ursache der megaloblastischen Entartung ist dabei nicht ein Vitamin B_{12}- oder Folsäure-Mangel, sondern eine andere tiefgreifende Störung im Zellstoffwechsel, was auch die Therapieresistenz gegenüber diesen Vitaminen eindeutig zeigt.

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Zusammenfassung

Es wird über Vitamin B_{12}-Bestimmungen im Serum bei 300 Patienten mit verschiedenen Erkrankungen berichtet. Charakteristisch niedrige Werte finden sich, abgesehen von der perniziösen Anämie, bei Polycythämie, Lymphadenosen, Morbus Waldenström, Schilddrüsenerkrankungen, Magenerkrankungen mit Anacidität und Darmerkrankungen. Bei Patienten mit Magenresektion zeigt der Vitamin B_{12}-Gehalt im Serum kein einheitliches Bild. Auffallend hohe Werte werden bei der chronischen myeloischen Leukämie gefunden. Dieser Befund läßt sich eventuell differential-diagnostisch gegenüber der Osteomyelosklerose verwerten, die normale oder niedrige Vitamin B_{12}-Werte aufweist. Erniedrigte Werte bei anderen Krankheitsgruppen ohne klinische oder hämatologische Manifestation eines Vitamin B_{12}-Mangels weisen auf die Häufigkeit passagerer Mangelzustände hin. Es wird betont, daß ein Vitamin B_{12}-Mangel nicht mit einer Hyperchromie einherzugehen braucht, wie auch hypo- oder normochromes Blutbild einen Vitamin B_{12}-Mangel nicht ausschließt.

Summary

Serum concentration of vitamin B_{12} in 300 patients with various diseases is reported. Apart from pernicious anaemia characteristically low values are found in polycythaemia, lymphadenosis, Waldenström's macroglobulinaemia, thyroid disease, gastric disturbances with anacidity and intestinal disorders. The vitamin B_{12} content in the serum of patients after gastrectomy is not consistent. Markedly high values are found with chronic myeloid leukaemia. This finding might be useful in the differential diagnosis from osteomyelosclerosis in which vitamin B_{12} levels are normal or low. Lowered values in other diseases without clinical or haematological evidence of vitamin B_{12} deficiency indicate the frequency of temporary deficiency conditions. It is emphasized that vitamin B_{12} deficiency is not necessarily associated with hyperchromia and that low or normal blood colour index does not rule out vitamin B_{12} deficiency.

Résumé

Le taux en vitamine B_{12} a été déterminé dans le sérum de 300 malades. Des valeurs basses caractéristiques se trouvent, l'anémie pernicieuse mise à part, dans la polycythémie, les lymphadénoses, la maladie de Waldenström, les maladies de la glande thyroïde, les maladies de l'estomac accompagnées d'anacidité et les maladies des intestins. Chez les malades ayant subi une résection de l'estomac, le taux sérique en vitamine B_{12} varie. Dans les leucémies chroniques, les valeurs sont élevées de façon frappante. Cette constatation peut fournir éventuellement une indication dans le diagnostic différentiel de l'ostéomyélosclérose qui, elle, présente des taux de vitamine B_{12} normaux. Des valeurs abaissées dans d'autres maladies sans manifestation hématologique révèlent la fréquence d'états de carence passagers. Il est insisté sur le fait qu'un manque en vitamine B_{12} n'est pas nécessairement accompagné d'une hyperchromie, tout comme une hypochromie ou une normochromie n'exclue pas un manque en vitamine B_{12}.

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Neonatal Thrombocytopenia in Association with Rubella

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Thrombocytopenic purpura has been found in association with many infections of the newborn period. In older children this hemorrhagic diathesis has frequently been associated with rubella. We have recently observed thrombocytopenia in seven newborns with the characteristics of the rubella syndrome and from whom German measles virus was cultured. The purpose of this report is to call attention to the relationship between thrombocytopenia and German measles infection in the newborn period.

Case Reports

Seven patients with purpura or other hemorrhage developing within 12 h of birth were seen by us in the newborn nursery from November 1964 to February 1965. The pertinent clinical and laboratory data on these infants and their mothers are shown in the Table. There was history of exposure to rubella or of rash, fever and lymphadenopathy suggestive of clinical rubella during the first trimester in six of the seven mothers. Two of these women received gamma globulin shortly after exposure. Neither thrombocytopenia nor platelet antibodies were present in the blood of any of the mothers. No evidence of blood group incompatibility was found. Most of the mothers received vitamins and iron medication during the antepartum period. Two were given diuretics during the last trimester of their pregnancies.

Five of the seven infants were of premature birth weight. In six cases the presenting symptom was purpura. The purpuric lesions were distributed over the body and face and were red, blue or violet in color Two patients had minimal gastrointestinal bleeding. Mild to moderate hepatosplenomegaly was found in five infants. Jaundice was seen in four patients, being mild in three and severe in one. Eye findings included bilateral and unilateral cataracts, transient glaucoma and retinal pigmentation. Evidence of heart disease was present in all but one patient. The cardiac abnormalities consisted of patent ductus arteriosus, auricular septal defect, ventricular septal defect, coarctation of the aorta and pulmonic stenosis, as diagnosed by clinical examination and catheterization. One patient was found later to be deaf.

Marked thrombocytopenia (<50000/mm^3) was present in all of these babies. All had normal hemoglobin levels, leukocyte counts and differentials. Three of the seven infants had increased normo-

We are indebted to Dr N. R. Shulman for performing the platelet antibody studies.

blast counts for their age. Bone marrow examinations uniformly showed hypocellularity and absent or markedly diminished numbers of megakaryocytes. Abnormal liver function tests were obtained in one patient. Direct and indirect Coombs tests and studies for toxoplasmosis, cytomegalic inclusion body disease and syphilis were consistently negative. Sepsis was excluded by the absence of positive blood cultures.

Rubella virus was cultured by the interference technique (1) from throat swabs of all the infants. The virus was also cultured in some cases from rectal swabs and urine.

Several cases required specific therapy for heart failure. Steroids were administered for the thrombocytopenia in only one instance. The duration of thrombocytopenia (<150000 platelets/mm^3) ranged from 7 to 30 days. In those cases where bone marrow examinations were repeated after recovery from the thrombocytopenia numerous megakaryocytes were found.

Death occurred in two infants, both from cardiac causes. Autopsy performed on one of these revealed patent ductus arteriosus, myocardial hypertrophy hepatitis, extramedullary hematopoiesis of the liver and spleen, and focal interstitial pneumonitis. Measles virus was grown from the heart of this patient.

Discussion

Within the past few years many of the causes of previously unexplained neonatal thrombocytopenia have been found. Although the newer syndromes include drugs (2) and immune mechanisms (3 4) intrauterine and neonatal infections probably account for many more cases. These include toxoplasmosis, cytomegalic inclusion disease, gram positive and negative septicemia (5) viral hepatitis (5) generalized herpes (6) and syphilis (7) With the exception of the mothers of two of our patients who received diuretics, none of the above causes was present.

Until recently only scattered cases (5 8, 9) of congenital thrombocytopenia were recorded in infants whose mothers had a history of clinical rubella in the first trimester Although viral studies were not performed in any of these previously reported cases, the infants showed the usual characteristics of the rubella syndrome. Recently RUDOLPH (10) has reported a number of newborns with rubella syndrome and thrombocytopenia from whom measles virus was cultured.

Table

Clinical and laboratory observations on seven infants with neonatal thrombocytopenia.

	Maternal history						
Case No.	Exposure to rubella	Clinical disease	Prenatal drugs	Sex	Birth weight	Purpura	Other hemorrhage
1	+	+	G.G.	M	3 lb 12 oz	+	None
2	+	None	G.G. /D.	M	5 lb 2 oz	+	None
3	+	+	D	F	6 lb 6 oz	+	None
4	+	+	None	F	5 lb 15 oz	None	Rectal
5	None	None	None	M	3 lb	+	G.I.
6	+	+	None	F	5 lb 1 oz	+	None
7	+	None	None	M	5 lb 4 oz	+	None

Gamma Globulin
Diuretics

In this report six of the seven mothers had a history of exposure to rubella and/or clinical manifestations during the first trimester. Various stigma of the rubella syndrome were noted in all of these infants. This report documents an additional seven cases of neonatal thrombocytopenia associated with the presence of rubella virus in newborns.

Decreased megakaryocytes were noted in the bone marrow specimens examined during the period of thrombocytopenia. Numerous megakaryocytes were found after the peripheral platelet counts returned to normal. From these observations it would appear that the cause of the thrombocytopenia is decreased megakaryocytopoiesis. The isolation of virus from the bone marrow of such patients which has recently been reported (11) is suggestive of direct effect on the megakaryocytes. In contrast, older children with thrombocytopenia following rubella have numerous megakaryocytes in their bone marrow. This would suggest a different mechanism for the platelet deficit in the latter situation.

Table (Continuation)

Hepatosplenomegaly	Jaundice	Platelets (mm³)	WBC (mm³)	Hemoglobin (gms. per 100 ml)	Bone marrow megakaryocytes	Throat culture for rubella virus	Duration of thrombocytopenic purpura
None	+	1 000	18 000	22	Decr	+	4 weeks
+	None	22 000	11 000	16	Decr	+	10 days
+	None	34 000	20 000	18	Decr	+	2 weeks
+	+	35 000	8 100	17	Decr	+	4 weeks
+	+	48 000	8 700	17	Decr	+	7 days
+	None	14 000	13 000	20	Decr	+	4 weeks
None	+	20 000	12 700	22	—	+	2 weeks

Although significant thrombocytopenia was present in every instance serious bleeding did not occur Therefore vigorous therapy such as steroid administration and platelet transfusions was not necessary Thrombocytopenia was self limited in all instances. The major problems of these patients centered around their cardiac defects.

It is interesting to note that hepatosplenomegaly was always present. Hepatitis, cardiac failure and extramedullary hematopoiesis may account for this enlargement.

Summary

Seven newborns with thrombocytopenic purpura and various characteristics of the rubella syndrome were observed In all of these cases the rubella virus was isolated from pharyngeal cultures The thrombocytopenia appeared to be due to decreased megakaryocytopoiesis and was of relatively short duration.

Résumé

7 nouveau-nés atteints de purpura thrombocytopénique et de différents symptomes caractéristiques d'un syndrome de roséole ont été observés. Dans chacun des cas, le virus de la roséole a pu être isolé du pharynx à l'aide de cultures. La thrombocytopénie semble avoir été due à une diminution de la mégacaryocytopoïèse et été de durée relativement courte.

Zusammenfassung

Es wird über 7 Neugeborene mit thrombocytopenischer Purpura und verschiedenen Erscheinungen des Rubeolen-Syndromes berichtet. Bei allen Fällen wurde aus den Kulturen der Rachenabstriche das Rubeolenvirus isoliert. Die Thrombocytopenie schien durch eine Verminderung der Megakaryocytopoese bedingt zu sein und war von relativ kurzer Dauer

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An Attempt to Separate Erythrocytes According to Age by a New Type of Centrifuge*

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Many facts speak in favour of the reticulocytes having larger cell volume but lower density than the erythrocytes. By simple sedimentation or conventional centrifugation the reticulocytes are concentrated in the top layer of the packed cells (1, 2, 3). The difference in the sedimentation rate has been ascribed to the difference in the density, but it has also been said that the inhability of the reticulocytes to form rouleaux is the main cause (3). The size of the particles is a dominating factor for sedimentation rate and erythrocyte aggregates have a more rapid one than single erythrocytes (4). Thus red cell separation is difficult, especially as the sedimentation rate is determined by many other factors than the density and the size of the cells (5).

It has been known for a long time that the red cells with malarial parasites are concentrated in the top layer by centrifugation (6), and that the parasites first attack the young red cells (7). It is also known that in pernicious anaemia with nucleated red cells (megaloblasts) in the blood, it is possible to concentrate these cells in the top layer by centrifugation (8). These and other observations have led to the conclusion that the red cells can be separated by centrifugation according to their age. Recently performed investigations support this hypothesis. For instance it has been shown that cholesterinase activity, which diminishes with the ageing of the red cells, is highest in the top layer of the packed red cells and lowest in the bottom layer (9). Investigations with the isotope Fe^{59} indicate the same thing. Thus after intravenous administration of the isotope of which the incorporation is limited

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to *the immature red cells* (10 11) the concentration is highest initially in the top layer increases in the bottom layer between 15th and 90th day after the administration and then decreases in the bottom layer after the 90th day (12) From these data the authors have concluded that the top middle, and bottom layers of centrifuged erythrocytes have young intermediate and old mean cell ages, respectively The decrease of the isotope activity in the bottom layer after 90 days is considered to be caused by the disappearance of senescent cells. Other investigations with the isotope Fe^{49} performed in the same way although ultracentrifugation was used gave the same result in principle (13)

Methods

For *separating the erythrocytes* new type of centrifuge constructed by Raftoelds (14) was used. This centrifuge is able to separate the particles into great number of fractions according to their sedimentation rate

Raftoelds described the principles of his centrifuges in the following y A suspension is run continuously through large number of intercommunicating shallow centrifuge tubes arranged either perpendicular to or (and) coaxial with the centre of rotation. A channel communicating with the centre of the centrifuge rotor is obtained by laying length of plastic tubing in spiral or circular groove or winding it around coaxial cylinder The peripheral wall of the groov or the inner wall of an outer casing (dependent upon the construction of the centrifuge) is provided with umber of ridges or thresholds. These ridges press the corresponding points on the peripheral wall of the tubing towards the centre and thus divide the tubing int large number of chambers or compartments, corresponding to large number of intercommunicating shallow centrifuge tubes. When suspension is continuously run through any one of the models, the heavier and larger particles sedimen t the beginning of the tubing and the lighter and smaller ones are carried farther w y and deposited more distally or peripherally The rotors contain large number of chambers or compartments and are constructed so that no particles escape from the peripheral open end of the tubing nd all of them are quantitatively caught and differen iated within short time

Using the Raftoelds centrifuge about 10 ml blood (w th an anticoagulant added) are needed. I order to get rid of the white blood cells which through forming aggregates spoil satisfactory separation of the red blood cells the blood samples first are centrifuged in an ordinary centrifuge for 10 minutes. After that, the supernatan and the top layer of the packed cells containing among others the hite blood cells is removed by suction. The remaining mass of red cells is resuspended into solution of physiological saline nd the suspension is dropped down into he centrifuge by tubing with drop counter

The type of Raftoelds centrifuge used is shown in Fig 1 The plastic ubing wound round the top and bottom bolts of the rotating interior cylinder so tha divided into L shaped compartments For centrifugation the author has used suspension of 2 ml red cells in 500 ml physiological saline dropped dow into the Raftoelds centrifuge t rate of one drop per second The quan ty nd concentration of the red cell suspension and the speed of the centrifuge has been chosen so that the greatest number of the erythrocytes are caught in the first 5 compartments (fraction I V After finishing the centrifugation which takes bout 1 hour w th this technique the packed cells are in the lower part of the L and he solution of physiological saline in the pper

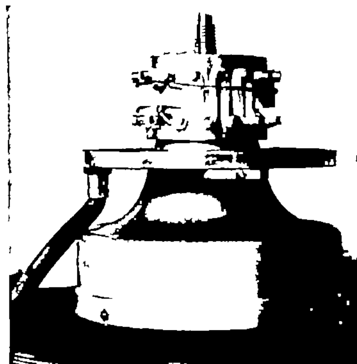


Fig. 1. The rotating interior cylinder of RAFFUALDI centrifuge. The plastic tubing is wound round the top and bottom bolts, so that it is divided into U-shaped compartments.

parts. The winding plastic tubing is cut off the upper bolts and the first 5 U' are centrifuged in an ordinary centrifuge during 15 minutes. After that, the shanks of the U' are cut off the border between the packed cells and the supernatant.

In these erythrocyte samples the number of cells in millions per ml (N) has been established in the ordinary manner in BÜRKER chamber though the dilution with Hayem solution has been 1 : 400. The mean corpuscular volume has then been calculated according to WINTROBE in μ^3 $\left(\frac{H \cdot 10}{N} = \frac{100 \cdot 10}{N} \mu^3\right)$. The haemoglobin weight in g corresponding to 100 ml of the erythrocyte mass (Hb) has been determined with Specker photometer for 20 ml of the erythrocyte mass haemolysed in 5 ml DRABKIN' solution. Then the mean corpuscular haemoglobin concentration (MCHC) has been calculated as $\frac{Hb \cdot 100}{H} = \frac{Hb \cdot 100}{100} = Hb$. Consequently with this technique the MCHC has the same numerical value as Hb. The mean corpuscular haemoglobin (MCH) has been calculated to 10^{-12} g.

The *reticulocyte percentage* has been established by counting 1000 red cells on smears stained with 0.5 solution of methylene blue.

For determination of HbF two different methods were used (15)

1. The present author employed the method of Kleihauer et al. (16). On smears treated according this method, the red cells with HbF have pink colour while the red cell with adult haemoglobin (HbA) because of the dissolving of HbA appear as colourless rings. In each fraction 1000 red cells were countered.

2. The same blood samples were examined by S. Bagdy according to the method of Betke (17) with slight modification of Bagdy (18). After haemolysis of the red cells the total amount of Hb and—after the dissolving of HbA—the amount of HbF was determined by Beckman quartz spectrophotometer.

Method 1 is below called the cell counting method and method 2 the denaturation method.

Material

The investigations have been made with human blood, both normal blood (cord blood and blood from adult blood donors) and blood from patients with iron deficiency anaemia and macrocytic anaemia. Furthermore an examination has been made of pig, horse and rabbit blood.

Results

Normal Human Cord Blood

Blood from the umbilical cord was withdrawn a few minutes after birth and before separation of the placenta. Twenty five infants were investigated. The data in Table I show that the percentage of reticulocytes increases successively from fraction I (0.2 ± 0.03) to fraction V (2.0 ± 0.4) and the difference is strongly significant ($P < 0.001$). (In 4 instances the percentage of reticulocytes was also established in fraction VI the mean value was 5.5 ± 0.6.) The results were as expected since fraction I corresponds to the bottom layer and fractions V and VI to the top layer obtained by ordinary centrifugation.

Table I
Normal human cord blood (25 cases)

Fraction (Kantgerkile)	R	MCV μ^3	MCHC	MCH 10^{-}
I	0.2 0.03	125 2.3	31.9 ± 0.3	39.7 0.7
II	0.2	120 2.0	30.6 ± 0.2	36.5 0.6
III	0.3	116 1.8	29.8 0.2	34.4 0.5
IV	0.6	112 ± 1.8	29.2 0.2	[illegible] 8 0.5
V	2.0 0.4	109 1.9	28.5 0.2	31.1 0.6

R = mean percentage value of the reticulocytes. MCV mean corpuscular volume. MCHC mean corpuscular haemoglobin concentration. MCH mean corpuscular haemoglobin.

The table shows how R increases whereas MCV, MCHC and MCH successively decrease from fraction I to fraction V.

Table I shows furthermore that the mean corpuscular volume (MCV) decreases successively from fraction I (125 ± 2.3) to fraction V ($109 \pm 1\ 9$) The difference is strongly significant ($P < 0\ 001$) and cannot depend upon the reticulocytes, as these have a greater volume than the erythrocytes and the number of reticulocytes increases from fraction I to fraction V Furthermore Table I makes clear that the mean corpuscular haemoglobin concentration (MCHC) also decreases successively from fraction I ($31\ 9 \pm 0.3$) to fraction V (28.5 ± 0.2) and this difference is also strongly significant ($P < 0\ 001$) As the difference also exists for the 4 infants where no reticulocytes were to be found in the first 5 fractions and the mean percentage of the erythrocytes for these fractions is very high (99 8 — 98 0) the reticulocytes cannot be the reason for the difference.

A phenomenon resulting from the decrease of MCV as well as MCHC from fraction I to fraction V is that the mean cell haemoglobin (MCH) also decreases (fraction I = $39\ 7 \pm 0\ 7$ fraction V = $31\ 1 \pm 0\ 6$) and the difference is strongly significant ($P < 0\ 001$)

The above mentioned investigations consequently have shown that as MCV decreases so does the MCHC, and a strongly significant positive correlation between MCV and MCHC has been pointed out ($P < 0\ 001$) (Fig 2)

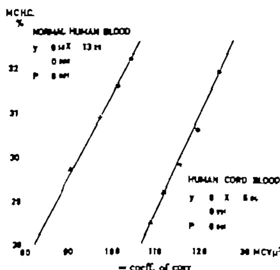


Fig 2 The diagram shows strongly significant positive correlation between MCV and MCHC in normal human cord blood and in normal human adult blood.

Table II
Normal human adult (10 cases)

Fraction (Reticulocytes)	R	MCV μ^3	MCHC	MCH 10^{-12}
I	0.2 ± 0.01	105 ± 1.8	32.2 ± 0.3	33.8 ± 0.6
II	0.3	102 ± 1.[illegible]	31.6 ± 0.3	32.1 ± 0.5
III	0.4	90 ± 1.2	30.9 ± 0.2	30.1 ± 0.4
IV	0.4	93 ± 0.9	30.3 ± 0.3	28.7 ± 0.4
V	0.8 ± 0.06	91 ± 0.7	29.7 ± 0.3	27.1 ± 0.4

Indications: Table I
The table shows in principle the same results as for the cord blood (Table I).

Normal Human Adult Blood

The blood samples have been examined immediately after drawing from 10 donors. As shown in Table II the investigation gave the same result as for the cord blood. The reticulocyte percentage increases from fraction I (0.2 ± 0.01) to fraction V (0.8 ± 0.06). The value of MCV on the other hand successively decreases (fraction I = 105 ± 1.8, fraction V = 91 ± 0.7) and so does the value of MCHC (fraction I = 32.2 ± 0.3, fraction V = 29.7 ± 0.3). Figure 2 shows a strongly significant positive correlation between MCV and MCHC ($P < 0.001$).

Blood from Patients with Anaemia

The investigations of blood samples from patients with iron deficiency anaemia (9 cases) and macrocytic anaemia (10 cases) will be presented here only as a correlation between MCV and MCHC. As seen from Fig. 3 the correlation between MCV and MCHC in the cases of iron deficiency anaemia is strongly significant positive ($P < 0.001$) and in the cases of macrocytic anaemia significant positive (P 0.01).

Blood from Normal Animals

Also here only the correlation between the value of MCV and MCHC will be presented. Blood samples from 9 pigs (aged 6 months), 8 rabbits (aged 2 months) and 9 adult horses have been investigated. As seen from Fig. 4 the correlation between MCH and MCHC in pigs is strongly significant positive (P 0.001) and in rabbits and horses significant positive (P 0.01).

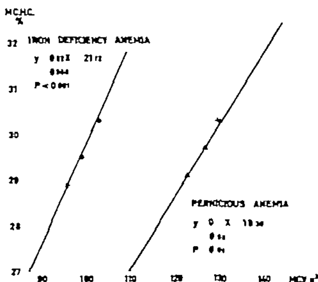


Fig. 3. The diagram shows significant positive correlation between MCV and MCHC in blood from patients with iron deficiency anaemia and pernicious anaemia.

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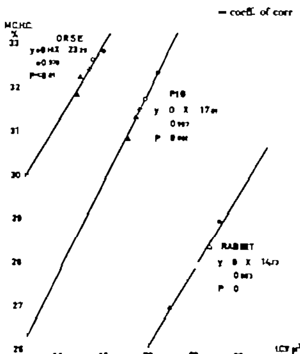


Fig. 4. The diagram shows significant positive correlation between MCV and MCHC in normal blood from horse, pig, and rabbit.

Table III
Normal human cord blood (9 infants)

Fraction (Rasterbild)	Cell counting method red cells containing HbF	Denaturation method HbF of total Hb
I	87.0 ± 1.6	85.0 ± 1.4
II	84.9 ± 1.9	81.9 ± 1.6
III	77.1 ± 3.2	79.4 ± 1.5
IV	70.0 ± 3.6	74.0 ± 2.8
V	64.7 ± 4.3	63.6 ± 5.1

The percentage figures obtained by the cell counting method are in near agreement with the corresponding figures obtained by the denaturation method. The figures decrease successively from fraction I to fraction V

The continued investigations were an attempt to make clear the age relation of the red cells in fractions I–V. As it has been maintained that young red cells are more resistant to haemolysis than older cells an analysis of the cell fragility in the different fractions was done. However, no conclusion could be drawn. More distinct results were obtained from the investigations of foetal haemoglobin (HbF) in normal human cord blood, planned according the principle—the larger the amount of HbF in a fraction, the older the red cells in the fraction. These investigations were carried out in collaboration with S. Brody (15).

The Amount of HbF in the Five Fractions

Cord blood from 9 normal infants was investigated by the two methods. Table III shows the results. The mean number of red cells with HbF is expressed as a percentage of the total number of red cells and the mean value of the amount of HbF as a percentage of the total amount of Hb. The percentage figures obtained by the cell counting method are in near agreement with the corresponding figures obtained by the denaturation method. They are largest in fraction I (with the cell counting method 87.0 ± 1.6, with the denaturation method 85.0 ± 1.4) and decrease successively from fraction I to fraction V. The corresponding figures in this fraction are 64.7 ± 4.3 and 63.6 ± 5.1 respectively.

By statistical analysis a strongly significantly positive correlation was obtained between the middle corpuscular volume and the amount of HbF determined by both the cell conting method ($P < 0.001$) (Fig. 5) and the denaturation method ($P < 0.001$) (Fig. 6).

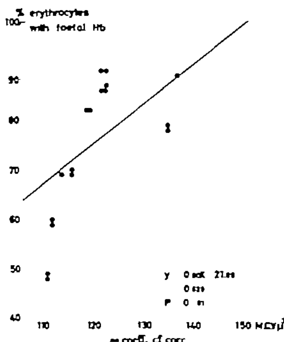


Fig 5. The diagram shows strongly significant positive correlation between MCV and the mean percentage value of the erythrocytes with foetal haemoglobin in normal human cord blood (see text).

As has been observed earlier some of the red cells in cord blood contain HbF as well as HbA (16) In our later investigation, therefore, the percentage figures in fraction I and fraction V for the red cells with 1 only HbF 2. HbF as well as HbA, and 3 only HbA were determined. Table IV shows the results. The number of red cells with only HbF is largest in fraction I (51 6 ± 2.3%) and smallest in fraction V (37 7 ± 1.5) while the contrary is the case for the red cells with only HbA (corresponding figures are 13 1 ± 2.3 and 31 1 ± 3 4) and the differences are strongly significant (P < 0 001) There is no significant difference for the intermediate group

Discussion

The examinations have shown that the erythrocytes in fraction I i e the erythrocytes with the most rapid sedimentation rate, have the greatest values for MCV and MCHC and consequently also for MCH The values successively decrease from fraction I to fraction V and by Student s t test a statistical significant positive correlation

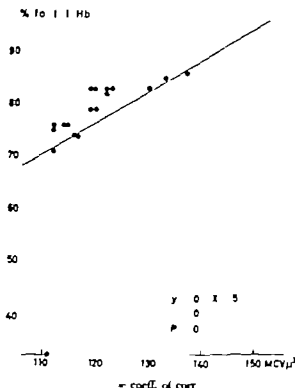


Fig 6. The diagram shows strongly significant positiv correlation between MCV and the mean percentage lue of the amoun of fœtal haemoglobi in normal human cord blood (see text)

Table IV
Normal human cord blood (9 infants)

Fraction ([illegible])	only HbF	red cells containing HbF and HbA	only HbA
I	51 6 2.3	35.3 _4	13.1 2.3
V	37 7 1 5	30 1 2.2	31 1 3 4

The percentage figures of red cells with only HbF re largest in fraction I nd smallest in fraction V while the contrary is the case for the red cells th only HbA.

between MCV and MCHC as well as MCH has been obtained in all groups.

Investigations of normal human cord blood have also shown that the greater the value of MCV in a fraction the greater the amount of HbF found in the fraction A strongly significant positive correlation between MCV and the amount of HbF has been obtained

The fact that the amount of HbF is greatest in fraction I indicates that the erythrocyte with the greatest sedimentation rate are the oldest red cells. Thus the investigation of HbF has given the same result as the isotope investigations which spoke in favour of the erythrocytes with the greatest sedimentation rate—and therefore by ordinary centrifugation obtained in the bottom layer—being the oldest red cells.

Thus the remarkable fact seems to exist that the amount of haemoglobin of the erythrocytes is correlated to the age in such a manner that the oldest erythrocytes have not only the greatest size but also the greatest amount of haemoglobin. As the formation of haemoglobin takes place only in the young red cells (10, 11), this fact is difficult to explain. A cause to the successively descending value for MCV from fraction I to fraction V may be a difference in the ability of old and young cells to form aggregates (3). However, the investigations performed with the above mentioned technique do not support this hypothesis and an increase of the value for MCHC by formation of aggregates is not very likely. The present author is disposed to relate the problem to the correlation between the degree of maturation of the red cells and their survival in the blood (19, 20, 21). This problem will be discussed further when the author has finished some comparative experiments by Rastgeldi and ordinary centrifuge techniques.

Summary

In blood samples from human beings and animals (pig, horse and rabbit) red cells were separated in a Rastgeldi centrifuge in such a manner that 5 fractions of erythrocytes with different sedimentation rates were recovered. The more rapid the sedimentation rate for an erythrocyte fraction the greater were its MCV, MCHC and MCH values in all groups investigated. In the 5 fractions of normal human cord blood there was a relation between the amount of foetal haemoglobin and the sedimentation rate of the erythrocytes.

Résumé

Dans le sang d'êtres humains et d'animaux (cochon, cheval et lapin) les érythrocytes ont été séparés à l'aide d'une centrifuge en 5 fractions ayant une vitesse de sédimentation différente. Plus la vitesse de sédimentation d'une fraction est grande, plus le volume cellulaire moyen, la concentration moyenne d'hémoglobine et le contenu moyen d'hémoglobine sont grands dans tous les groupes étudiés. Dans les 5 fractions de sang humain normal provenant du cordon ombilical, il existe une relation entre la quantité d'hémoglobine foetale et la vitesse de sédimentation des érythrocytes.

Zusammenfassung

Im Blut vom Menschen und von Tieren (Schwein, Pferd, Kaninchen) wurden die Erythrocyten mit der Rastgeldi-Zentrifuge in 5 Fraktionen mit verschiedener Sedimentationsgeschwindigkeit aufgeteilt. Je schneller die Sedimentation in einer Fraktion war, um so größer waren bei allen untersuchten Gruppen das mittlere Zellvolumen, die mittlere Hämoglobinkonzentration und der mittlere Hämoglobingehalt der roten Blutkörperchen. In den 5 Fraktionen von normalem menschlichem Nabelschnurblut fand sich eine Beziehung zwischen dem Gehalt an fötalem Hämoglobin und der Sedimentationsgeschwindigkeit der Erythrocyten.

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A Simplified Procedure for Brewer's Methemoglobin Reduction Test*

A. Tizianello, I. Pannacciulli and E. Salvidio **

In 1960 Brewer *et al.* (2) introduced their methemoglobin reduction test as a simple screening method for the detection of G-6-PD deficiency in red blood cells. This method is based on the oxidation of hemoglobin to methemoglobin by sodium nitrite and the subsequent reduction to hemoglobin by methylene blue through TPNH linked MetHb-reductase. Applied to large scale screening of a great number of cases it has prooved highly sensitive and satisfactory (3, 4, 7, 8).

We have performed more than 4000 Brewer's tests, finding this method simple, reliable and inexpensive. The modifications here described are intended to overcome some of its disadvantages, preserving at the same time its high sensibility.

Material and Methods

The original methemoglobin reduction test introduced by Brewer *et al.* [illegible] can be summarized as follows: to [illegible] ml of blood, freshly collected in ACD, 0.1 ml of 0.18 M sodium nitrite solution and 0.1 ml of 0.0004 M methylene blue solution are separately added. After 12 gentle inversions the test tubes are incubated at 37 °C in [illegible]. At [illegible] and 170 minutes the blood is mixed by blowing one breath of air gently through a 1 ml pipette. At 180 minutes 0.1 ml of the red cells are hemolyzed in 10 ml of 0.0[illegible] M phosphate buffer (pH 6.6) and photometric readings of the remaining methemoglobin before and after the addition of [illegible] drop of 0.4 M sodium cyanide are taken at 640 nm. The total Hb on the same sample is determined by transferring 2 ml of the hemolysate to 8 ml of 0.0[illegible] M phosphate buffer (pH 6.[illegible]); one drop of 0.6 M potassium ferricyanide and one drop of 0.4 M sodium cyanide are added to this second solution and the readings are taken at 540 nm.

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** Principal Investigator, P.H.S. Grant HE-[illegible].

The following modifications have been introduced

(1) Sodium nitrite in distilled water and methylene blue in sodium chloride solution were pipetted in dark vials of 5 ml capacity lyophilized, filled with nitrogen and sealed. At the end of the lyophilising procedure each vial contained 0.374 mg of methylene blue, 33 mg of sodium nitrite and 20.25 mg of sodium chloride. The vials, kept in the dark, can be stored for several months without alterations in the activity of the reagents. Immediately before its use the content of each vial was dissolved into exactly 5 ml of distilled water 0.1 ml of the solution was pipetted into the test tube containing 1 ml of blood.

(2) A rapid twelvefold inversion of several test tubes at the same time was made possible by the use of rigid pad, having the surface of 20 test tubes rack, and which was covered with washable rubber sheet.

(3) The amount of both buffer solutions necessary for the methemoglobin and hemoglobin readings was reduced respectively to 5 ml and 4 ml 0.1 ml of blood was transferred from the test tube to the first buffer solution and 1.0 ml of the hemolysate was thereafter transferred to the second buffer solution.

In order to overcome the disadvantage of the long (3 h) incubation time, set of experiments was performed substituting Nile blue (0.1 ml of 14.5% solution) for methylene blue. Owing to the faster recovery rate of methemoglobin to oxyhemoglobin induced by the first dye (1) the incubation time was then reduced to two hours.

Blood of normal and G-6-PD deficient subjects was screened. G-6-PD deficient cases were controlled, performing G-6-PD assays according to KORNBERG AND HORECKER (6).

Results

(1) A comparison between the amount of methemoglobin remaining in the tested blood of 57 normal and 58 mutant subjects checked at the same time with the original Brewer's test and the vial method is summarized in Table I The χ^2 test for paired observations was employed in the statistical analysis. The difference between the two groups was not statistically significant. In the second group there was no case which had abnormal methemoglobin values with the original Brewer's test and yielded normal values with the vial method.

(2) In 4059 normal cases screened by means of the original Brewer's test the mean percent of remaining methemoglobin was 3.88 in a group of 264 normal subjects tested by the modified vial method the mean percent of remaining methemoglobin was 3.77

(3) In the experiments carried out with Nile blue the results obtained in normal cases were comparable with those of the original Brewer's test, whereas the determinations performed on intermediate G-6-PD mutants were unreliable, owing to false negative results (Table II)

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We have performed more than 4000 Brewer's tests, finding this method simple, reliable and inexpensive. The modifications here described are intended to overcome some of its disadvantages preserving at the same time its high sensibility.

Material and Method

The original methemoglobin reduction test introduced by Brewer *et al.* (2) can be summarized as follows: to 2 ml of blood, freshly collected in ACD, 0.1 ml of 0.18 M sodium nitrite solution and 0.1 ml of 0.0004 M methylene blue solution are separately added. After 12 gentle inversions, the test tubes are incubated at 37 °C in a water-bath. At 60 and 120 minutes the blood is mixed by blowing one breath of air gently through a 0.1 ml pipette. At 180 minutes 0.1 ml of the red cells are hemolyzed in 10 ml of 0.02 M phosphate buffer (pH 6.6) and photometric readings of the remaining methemoglobin before and after the addition of one drop of 0.4 M sodium cyanide are taken at 640 nm. The total Hb on the same sample is determined by transferring 2 ml of the hemolysate to 8 ml of 0.02 M phosphate buffer (pH 6.6); one drop of 0.6 M potassium ferricyanide and one drop of 0.4 M sodium cyanide are added to this second solution and the readings are taken at 540 nm.

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** Principal [illegible] P. H. S. Grant HE 5682.

The attempts made to replace methylene blue with Nile blue in order to shorten the incubation time of the mixture (point 6) have failed, owing to the false negative results which were frequently obtained on the blood of intermediate subjects, which had been checked also by G-6-PD assays.

In our opinion the above listed modifications of Brewer's test may increase its routinary exploitation in clinical laboratories and blood banks. As a matter of fact the detection of G-6-PD deficiency in blood donors should be currently pursued in view of the possibility of unexpected hemolytic reactions following the administration of certain drugs in recipients of G-6-PD deficient blood (9)

Summary

Several modifications of Brewer's methemoglobin reduction test are described. Among them, the lyophilization of both sodium nitrite and methylene blue solutions into single vial improves the stability of these reagents. These modifications, extensively checked, do not impair the sensibility of the methemoglobin reduction test.

Résumé

Plusieurs modifications du test de la réduction de la méthémoglobine d'après Brewer sont décrites. Parmi celles-ci la lyophilisation du nitrite de sodium et du bleu de méthylène dans le même récipient améliore la stabilité des réactifs. Ces modifications ne diminuent pas, selon les épreuves étendues auxquelles elles ont été soumises, la sensibilité du test de réduction de la méthémoglobine.

Zusammenfassung

Es werden verschiedene Modifikationen des Methämoglobin-Reduktionstestes nach Brewer beschrieben. Dabei lässt sich durch Lyophilisierung der Lösungen von Natriumnitrit und Methylenblau im gleichen Gefäß die Stabilität dieser Reagentien verbessern. Eine ausgedehnte Prüfung ergab, daß die Modifikationen die Empfindlichkeit des Methämoglobin-Reduktionstestes nicht beeinträchtigen.

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Promyelocytic Leukaemia and Hypofibrinogenaemia

R. J. W. RYDER

Bleeding during the course of leukaemia may be troublesome and the reason for it obscure. CROIZAT AND FAVRE-GILLY (7) considered that there was insufficient information to determine the cause of bleeding in patients with leukaemia, and investigated the coagulation mechanism routinely in their patients. They found that bleeding was usually associated with thrombocytopenia, and that the fibrinogen level was normal or even raised. However they described one instance of the unusual association of hypofibrinogenaemia with bleeding in a patient with myeloid leukaemia. Prior to this RISAK (26) had reported fibrinogenopenia in a patient with myeloblastic leukaemia. Other reports have been accumulating, and now there are records of about 60 patients with hypofibrinogenaemia and myeloid leukaemia. ROSENTHAL (27) believes that there is now sufficient evidence for regarding 'promyelocytic fibrinopathic leukaemia' as a distinct syndrome. Characteristic features of this syndrome are the presence of abnormal promyelocytoid cells in the bone marrow and an unexplained hypofibrinogenaemia. A rapid downhill course, little influenced by therapy with severe haemorrhagic manifestations, culminating in death from either gastrointestinal or cerebral haemorrhage, is commonly seen. Lymphadenopathy hepatomegaly and splenomegaly are unusual, and when present are slight. This report deals with another patient with this syndrome.

Methods

Routine haematological methods (8, 9) were employed. For platelet counts the method of MACFARLANE AND BROOS (19) was used. The one-stage prothrombin time was measured using human brain thromboplastin. The prothrombin and proconvertin estimation was carried out by the method of OWREN AND AAS (24). The thromboplastin generation screening test (15) was performed using Inosithin (17) and Bell & Alton chloroform extract of acetone-dried human brain as platelet substitutes. Prothrombin,

factor V and factors VII and X were measured by the methods of ALAGILLE (1) and factor X by the technique of DENSON (10). Fibrinogen was estimated by the method of I RAM (17), and subsequently by electrophoresis of plasma and serum, by measurement of the turbidity after addition of calcium-thrombin solution (15) by measurement of the turbidity after addition of ammonium sulphite (18) by measurement of the coagulated protein after heating at 56 °C for 10 minutes, and by an immunological method (Fi-test, Hyland Laboratories). Fibrinolysis was sought by observation of the clot in whole blood for 48 hours, and observation for 72 hours of the clots in serial dilutions of citrated plasma in buffered saline, after the addition of human thrombin in one series and recalcification of the other the tubes being kept stoppered in a water-bath at 37 °C.

Case History

A 73 year old woman was admitted from a home for patients with senile dementia with a history of intermittent epistaxes during the preceding month. She had lost several ounces of blood on the previous day but her bleeding had ceased before admission. There was no history of previous bleeding or abnormal bruising. She had had respiratory infection, and prior to admission had received intramuscular penicillin and streptomycin, and had also been given digoxin and chlorpromazine.

On examination she was pale, without cyanosis or jaundice. Her temperature was 97 F Occasional scattered petechiae were seen. There was extensive bruising of her buttocks and thighs, and ecchymoses were present on the backs of her hands. Her liver and spleen were not palpably enlarged. There was no superficial lymphadenopathy Bone tenderness was bsent. Examination of her respiratory system revealed signs of emphysema without evidence of infection. Her blood pressure was 160/90 mm Hg, and auscultation of her heart revealed slow atrial fibrillation and an apical systolic murmur There were no other significa t findings. The results of a blood count are shown in Table I. These findings suggested a diagnosis of promyelocytic leukaemia which was confirmed by bone marrow biopsy carried out on the right iliac crest under local anaesthesia. The normal marrow structure was largely replaced by primith cells of the granulocyte series, many of which were abnormal, having large irregular nuclei with fine chromatin pattern suggesting immaturity while the cytoplasm contained large chunky neutrophilic granules. Occasional giant metamyelocytes and macropolycytes were present—however erythropoiesis was normoblastic. Few megakaryocytes were present.

Eigh hours after the bone marrow biopsy considerable oozing from the site of puncture was noted. Investigation of her coagulation mechanism gav the following findings. The bleeding time (DUKE) was greater than 25 minutes. The whole blood coagulation time in plain glass was 10 minutes. Capillary resistance (tourniquet test) was not decreased. The one-stage prothrombin time was prolonged, and the addition of Russell viper venom did not correct this abnormality A thromboplastin generation screening test gav normal result. Assay of her circulating coagulation factors gave the results shown in T ble II, a. Evidence of fibrinolysis was not detected.

A transfusion of two pints of fresh blood was given, and treatment with prednisolone (1?0 mg per day) 6-mercaptopurine (100 mg per day) and epsilon-amino-caproic cid (.0 g orally per day) was commenced. The oozing from her puncture site wa controlled by local measures and she remained free from external bleeding During the following days her general condition improved little However her leucocyte count rose progressively and on the ninth day after admission her condition worsened. A further study of her coagulation mechanism was carried out, the results of which are shown in Table II, b. She deteriorated rapidly and died later that day

Table I

	30th June	3rd July	6th July	8th July
Haemoglobin, g/100 ml	7.9	5.3	8.6	6.4
Leucocytes per mm³	8 850	15 900	41 600	35 000
Platelets per mm³	47 000	50 000	71 000	58 000
Reticulocytes, %	3.6	3.2	2.3	3 6
Neutrophils, %	70	55	72	79
Metamyelocytes, %	1	2	1	1
Myelocytes, %	1	1	1	1
Promyelocytes, %	17	29	22	14
'Blasts %'	3	2	1	1
Lymphocytes, %	8	11	3	3
Monocytes, %	—	—	—	—
Eosinophils, %	—	—	—	1

Table II

		b
Prothrombin complex, %	39	28
Prothrombin and proconvertin, %	42	50
Prothrombin, %	25	40
Factor V %	80	53
Factor VII and X, %	48	90
Factor X, %	100	100
Thromboplastin generation screening test	normal	normal
Fibrinogen, mg%	85	30
Fibrinolysis	absent	absent

Autopsy findings. At autopsy surprising absence of post-mortem clotting was noted. A small subendocardial extravasation of blood was present in the right atrium. Small subendothelial ecchymoses were present under the left parietal pleura. The bronchial walls were congested, and the larynx, trachea and bronchi contained some blood-streaked sputum and some altered blood. Submucosal ecchymoses were present in the lower third of the oesophagus. The stomach did not contain blood, but altered blood was present in the duodenum and the small and large intestines as far as the descending colon. The sites of bleeding could not be identified. Scattered ecchymoses were present under the peritoneum of the pelvis.

The lungs showed emphysema with basal congestion and slight oedema. Scarring was present at the right apex, which histological examination showed to have resulted from previous tuberculous infection.

The heart weighed 270 g, enlargement being due to concentric hypertrophy of the left ventricle which measured 17 mm. The right coronary artery had minute lumen just beyond its origin. Microscopic examination showed recanalization of previous thrombus.

The liver weighed 1020 g. The cut surface showed congestion but was otherwise normal in appearance. Microscopically increased numbers of granulocytes were present in the portal tracts.

The spleen was of normal size, weighing 110 g, with smooth slate grey capsule. On section it was soft, congested, and with loss of its follicular pattern. Microscopically it showed congestion, with diffuse increase in granulocytes in the red pulp.

A few small lymph nodes were present at the hila of the lungs and in the mesentery. The hilar nodes contained anthracotic pigment but were otherwise normal. The mesenteric nodes contained small collections of granulocytes, most prominent in the subcapsular sinuses.

The kidneys were diminished in size, the right weighing 100 g, the left 110 g. An atheromatous plaque at the origin of the left renal artery caused narrowing of its lumen. Histological examination showed evidence of healed pyelonephritis and benign nephrosclerosis.

The total weight of the adrenal glands was 8 g and there was marked depletion of cortical lipid.

The pancreas was normal on gross and histological examination.

Bone marrow was hyperplastic and showed a marked increase in cells of the granulocyte series, with a predominance of early forms.

There was no histological evidence of microthrombi in any organ.

Discussion

Type of Cells

CROIZAT AND FAVRE-GILLY (7) recorded that the bone marrow of their patient was replaced by myeloblasts and promyelocytes, and HILLESTAD (16) noted a marked increase in the proportion of promyelocytes in the bone marrow biopsy specimens from two patients, but none of these workers commented on the fine structure of these cells. In a more recent report, BERNARD *et al.* (5) observed that the nuclear chromatin of the promyelocytes was denser than in myeloblasts but not so dense as in normal promyelocytes. Cytoplasmic granules were increased in number and usually in size also. ROSENTHAL (27) pointed out that there was dyskinesia between the maturation of the nucleus and the cytoplasm, the nucleus being relatively immature whereas the cytoplasm showed signs of maturity in its loss of basophilia and content of granules. These granules varied in size, shape and staining characteristics, although within any one cell the granules had the same appearance. He suggested that the cells should be termed promyelocytoid. DIDISHEIM *et al.* (11) recorded large PAS-positive cytoplasmic granules in the promyelocytes of one of their patients. Abnormal promyelocytoid cells with prominent granules were present in increasing numbers in this patient's peripheral blood during her brief illness. The PAS-positive granules recorded by DIDISHEIM *et al.* could not be demonstrated.

Hypofibrinogenaemia

The hypofibrinogenaemia, a characteristic feature of this syndrome and present in this patient, is difficult to explain. Pos-

sible explanations are 1 decreased synthesis, 2. loss, 3 alteration 4. excessive utilisation, 5 fibrinolysis.

1 Decreased synthesis Tests of liver function did not reveal any abnormality in ROSENTHAL's patients. Findings in the patient reported here are Total serum proteins 6.1 g%, of which albumin formed 3.5 g and globulin 2 6 g Bilirubin —direct reacting—nil, indirect reacting 0.8 mg% Thymol turbidity 2 units. Zinc sulphate flocculation 3 units. Alkaline phosphatase 1 7 K.A. units. Serum glutamate-oxalacetate transaminase 27 units. Serum glutamate pyruvate transaminase 20 units. Although these tests are relatively insensitive they do not reveal any gross abnormality and the near normal albumin level suggests that protein synthesis is not gravely impaired and diminished production of fibrinogen an unlikely explanation.

2 Loss There was no evidence of protein loss either into the body cavities or externally A loss of albumin, because of its smaller molecular size, would be expected to precede a loss of fibrinogen.

3. Alteration Estimation of fibrinogen by a technique in which fibrin is the product measured would give erroneously low results if there were interference with the conversion of fibrinogen to fibrin. BAKER AND JACOB (2) described a haemorrhagic disorder in pregnancy due to an anticoagulant' which prevented the conversion of fibrinogen to fibrin. FLETCHER *et al.* (14) reported that the presence of fibrinogen digestion products may interfere with fibrin polymerisation. High concentrations of urea may have an inhibitory effect on the polymerisation of fibrinogen (28) However ELLIS AND STRANSKY (13) found that artificially raising the plasma urea to 500 mg% did not influence the result in the estimation of fibrinogen, and concluded that interference by urea within circumstances likely to be encountered clinically was impossible.

The finding of a low circulating fibrinogen level by 6 techniques, associated with deficiencies of other circulating coagulation factors, suggests that a true fibrinogenopenia was present in this patient, whose blood urea was 70 mg%.

4 Excessive utilisation. Massive intravascular clotting of the type found in the fibrination-defibrination syndrome could explain the increased clearance of fibrinogen from the circulation found by DIDISHEIM *et al.* (11) The defibrination syndrome is associated with thrombocytopenia, fibrinogenopenia, and a variable reduction of other circulating coagulation factors, all of which may be found in

promyelocytic leukaemia also Deficiencies of factors II V VII VIII IX and X in varying combinations have been reported in promyelocytic leukaemia by NILSSON *et al.* (22) BERNARD *et al.* (5) and ROSENTHAL (27) of which factor V is the most constantly and the most severely affected. Yet ROSENTHAL administered heparin to one of his patients without improvement in the levels of fibrinogen or factor V, or diminution in the rate of clearance of fibrinogen from the circulation.

Intravascular coagulation and defibrination could explain the deficiencies of circulating coagulation factors found in this patient. The one discordant feature is the diminished levels of factors VII and X, with a normal level of factor X when this was estimated separately indicating a decreased level of factor VII This would be unexpected in serum—which is what the plasma of a patient with defibrination syndrome resembles.

MERSKY *et al* (21) reported a patient with hypofibrinogenaemia secondary to disseminated colonic cancer and concluded from their studies that systemic defibrination and locally induced fibrinolysis coexisted. Reduced levels of prothrombin factor V factor VIII and platelets were considered evidence of defibrination, while reduced levels of plasminogen, streptokinase and urokinase inhibitors, together with a prolonged thrombin clotting time, were considered to indicate fibrinolysis. Parenteral administration of heparin sequentially corrected defibrination and secondary fibrinolysis. Discontinuance of heparin resulted in recurrence of intravascular coagulation and reactivation of the fibrinolytic process.

5 Fibrinolysis An increase in fibrinolytic activity has been noted in the majority of reported cases of promyelocytic leukaemia. Its source is uncertain HILLESTAD (16) states that promyelocytes contain trypsin, which activates plasminogen and also has a direct fibrinolytic effect, and considered that this may be responsible ROSENTHAL (27) reported that a remission in one patient, with disappearance of the promyelocytes from the bone marrow was associated with a rise in the fibrinogen level. DIDISHEIM *et al* (11) reported similar findings, with a subsequent fall in the fibrinogen level accompanying a terminal rise in the promyelocyte count. ROSENTHAL could not demonstrate fibrinolytic material in an extract prepared from promyelocytes. In the largest series of reported cases, BERNARD *et al* (4) demonstrated increased fibrinolysis in 11 of 15 patients in whom it was sought.

Fletcher *et al.* (14) noted that plasmin, in addition to degrading fibrinogen and fibrin, also degrades other specific coagulation factors. They mention that there is agreement that factors V and VIII may be destroyed, but disagreement as to whether prothrombin, factor VII and factor IX are susceptible to enzymatic degradation. Coon and Duff (6) using the method described by Owren and Aas (24) observed decreased factor V and factor VII activity after intravenous infusion of plasmin in dogs. Hillestad (16) who also used Owren and Aas's method, stated that plasmin may digest prothrombin, factor V and factor VII. McNichol *et al.* (20) reported decreased fibrinogen, prothrombin, factor V and factor VIII with a slight inconstant fall in factor IX, but without alteration in the levels of factors VII and X, in patients who had received intravenous infusions of streptokinase. Donaldson (12) demonstrated reduction of factor V factor VIII and factor IX following the incubation of normal plasma with plasmin, but failed to detect any diminution in factors VII, X, XI and XII. In a further experiment in which normal plasma was incubated with streptokinase, she found a constant decrease only in factor V and concluded that full activation of plasmin by streptokinase does not occur in unclotted plasma. Fletcher *et al.* (14) report that, in patients treated with streptokinase, the factor V concentration fell, but that neither the prothrombin concentration nor the platelet count was reduced.

In the patient reported here, the levels of prothrombin, factor V and factor VII were decreased. The platelet count also was reduced, but few megakaryocytes were to be seen in the bone marrow and the thrombocytopenia may well have been due to a failure of production.

Nilsson (23) reported that the administration of epsilon-amino-caproic acid in a dose of 36 g orally per day to a patient in whom fibrinolytic activity was high brought about a disappearance of fibrinolytic activity. Increased fibrinolysis was not demonstrated in the patient reported in this paper in whom a rising promyelocyte count was associated with a falling fibrinogen level in spite of the administration of epsilon-amino-caproic acid.

However Sherry *et al.* (29) state that increased fibrinolytic activity is not necessarily a prerequisite for the diagnosis of a fibrinolytic disorder since the factors responsible for this activity may be gone from the circulation by the time the study is made, while their

effects are demonstrable. Thus digestion by plasmin could explain the deficiencies found in this patient, while fibrinolytic activity remained undetected by the methods employed. If this is the explanation it is surprising that epsilon-amino-caproic acid did not block the activation of plasminogen and bring about an increase in the level of fibrinogen and other circulating coagulation factors. Perhaps the explanation is to be found in the statement of SHERRY *et al.* that in the late stages of some leukaemias, proteolytic enzymes other than plasmin may appear in the circulation and produce a 'hyper proteolytic state

BAKER *et al.* (3) reported a patient with acute myelogenous leukaemia who had severe bleeding associated with hypofibrinogenaemia. Reduced levels of factor V factor VIII and fibrinogen were consistent with either primary defibrination or primary fibrinolysis. Thrombocytopenia associated with a normal number of megakaryocytes in the bone marrow normal euglobulin lysis time normal antiplasmin levels and normal antithrombin activity were in favour of defibrination, but the normal prothrombin level and diminished plasminogen value were more in accord with primary fibrinolysis. Factor VII was reduced to 68 in this patient, but no comment made as to the significance of this. Administration of epsilon-amino-caproic acid and fibrinogen was without benefit. Heparin brought about a rise in the levels of fibrinogen, factor V and plasminogen, and accompanying transient clinical improvement. A second course of heparin was without benefit. Subsequent autopsy revealed evidence of widespread intravascular coagulation.

So it may be seen that in patients with promyelocytic leukaemia and accompanying hypofibrinogenaemia, the elucidation of the underlying mechanism(s) may be difficult. A further complicating feature is that patients with leukaemia who have an increased susceptibility to infection may have a disturbance of coagulation secondary to this. A report of coagulation studies in a patient with fatal Pseudomonas septicaemia by RAPAPORT *et al* (25) is of interest. In their patient, diminished levels of fibrinogen, factor V and factor VIII in the absence of evidence of fibrinolysis, were considered evidence of extensive intravascular coagulation. Decreased levels of factors VII IX, X and XI were more difficult to explain, but these workers noted that a fall in the serum factors had been documented in rabbits given two injections of endotoxin, and suggested that endotoxin might have been responsible in their

patient for the decreased levels of circulating coagulation factors. Histological examination of the kidneys of their patient disclosed the widespread deposition of material staining like fibrin within the glomerular vessels. They believe that these findings show that bacterial endotoxin may induce a generalised Schwartzman reaction in man. However in the patient reported in the present paper there was no evidence either during life or at autopsy of generalised infection.

In summary this patient with promyelocytic leukaemia had decreased levels of prothrombin, factor V factor VII, fibrinogen and platelets. Few megakaryocytes were seen in her bone marrow and her thrombocytopenia can be attributed to a failure of platelet production. The decreased levels of fibrinogen and factor V could have been due to either defibrination or fibrinolysis. The decrease in prothrombin is more suggestive of defibrination. The lowered level of factor VII is surprising, as an increase or an unchanged level would be expected in defibrination, and opinion is divided as to whether factor VII is decreased or not in fibrinolysis.

Acknowledgement. I should like to thank Dr J. R. MAHON for permission to study this patient under his care and for helpful advice in the preparation of this paper

Summary

A patient with acute promyelocytic leukaemia and accompanying hypofibrinogenaemia is described. Decreased levels of prothrombin, factor V factor VII and platelets were present. Evidence of fibrinolysis was not detected and administration of epsilon-amino-caproic acid failed to bring about any improvement. The underlying mechanisms which may have been responsible for the hypofibrinogenaemia are considered.

Résumé

Description du cas d'un malade atteint d'une leucémie promyélocytaire aiguë. Une diminution du taux de prothrombine, de facteur V et VII ainsi que du nombre des thrombocytes était présente. Une fibrinolyse ne put pas être mise en évidence et l'application d'acide epsilone-amino-caproïque n'apporta aucune amélioration. Les mécanismes pouvant avoir mené à l'hypofibrinémie sont discutés.

Zusammenfassung

Es wird über einen Patienten mit akuter Promyelocytenleukämie und Hypofibrinogenämie berichtet. Prothrombin, Faktor V Faktor VII und Plättchenzahl waren vermindert. Eine Fibrinolyse war nicht nachzuweisen, und die Zufuhr von Epsilon-aminocapronsäure war wirkungslos. Die pathogenetischen Mechanismen der Hypofibrinogenämie werden erörtert.

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D. J. Weatherall, **The Thalassaemia Syndromes.** Blackwell Scientific Publ. Oxford 1965. 272 S. Preis 45 s.

Die Monographie vermittelt eine vollständige Übersicht über die Thalassämie-Varianten, über die mit einer Thalassämie kombinierten Hämoglobinanomalien und die differentialdiagnostisch wichtige hereditäre Hb F Persistenz. Neben klinischen Fragen werden Probleme der Pathogenese und der Genetik besprochen.

Nach einer kurzen historischen Orientierung wird zuerst die genetische Kontrolle der Hämoglobinsynthese und die Bedeutung der Thalassämie erläutert. Dann folgen Kapitel über die verschiedenen Formen der β-Thalassämie mit speziellen Abschnitten über die δ-Ketten-Thalassämie, über die Sichelzell-Thalassämie und die Kombination der β-Thalassämie mit den anomalen Hämoglobinen C, E, J, N, D, G und L. Ein besonderes Kapitel ist den Hb Lepore-Anomalien gewidmet, die Musterbeispiele einer thalassämieähnlichen Störung mit genauer bekanntem Defekt darstellen. Der β-Thalassämie wird sodann die andersgeartete Anomalie der hereditären Hb F Persistenz gegenübergestellt. Nachher werden die diagnostischen und pathogenetischen Probleme der α-Thalassämie abgehandelt. Ein selbständiges Kapitel betrifft klinische Gesichtspunkte der Diagnostik und Therapie. Es enthält u. a. nützliche diagnostische Hinweise für den Arzt, technische Angaben über die Bestimmung der normalen und anomalen Hämoglobine sowie Richtlinien über die Indikation zur Splenektomie und die Eisenentfernung aus dem Organismus. Den Schluss bilden eine kurze Übersicht über genetische und molekularbiologische Grundlagen der Thalassämie und ein Ausblick auf Zukunftsprobleme.

Das handliche Buch ist übersichtlich gegliedert, leicht lesbar und enthält für jedes Kapitel eine knappe Zusammenfassung. Zahlreiche Tabellen, Diagramme und gute Abbildungen sowie ein ausführliches Literaturverzeichnis erhöhen den Wert der Monographie, die jedem Hämatologen sehr empfohlen werden kann. H. R. Marti, Basel

Oscar W. Schalm, **Veterinary Hematology.** Lea & Febiger, Philadelphia 1965. 2nd edition. 84 illustrations on 14 plates and 30 in color on 7 plates. Price $ 15.–.

The second edition of this book (see review A.H. 28, 1962, p. 71) is greatly enlarged, good color plates are added. A great deal of additional useful and interesting information has been supplied e.g. autoimmune hemolytic anaemia in dogs, indications for B12 therapie in dogs with megaloblastosis. The color plates, taken from other publications, are well reproduced. The inclusion of normal blood values for guinea-pig, hamster, monkey, rat and mouse will be helpful for everyone working in experimental haematology. On the whole, the book can also be highly recommended to the "human" hematologist. O. Rosenow, New York

Aaron J. Marcus and Marjorie B. Zucker, **The Physiology of Blood Platelets.** Grune & Stratton, Inc., New York/London 1965, 162 pp. Price $ 7.–

The authors present a comprehensive review on the more recent biochemical, morphologic and clinical research on platelets. Biochemistry, coagulation factors associated with platelets, thrombocytopathia, electron-microscopy haemostasis, thrombosis, thrombasthenia, thrombopoesis and platelets transfusions are some of the discussed topics. 875 references are given. O. Rosenow, New York

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On Standardization in Haematology *

Ch. G. de Boroviczény

In the past few years considerable effort has been directed toward standardizing haematological methods on an international level. The lively response to these activities proves how very necessary are such measures.

During the course of a round table discussion at the European Congress of Haematology in Vienna in 1961 the urgent demand for international regulations became particularly evident and haematologists in Germany (Boroviczény, Heilmeyer) joined forces with colleagues from the Netherlands (Coster, Holtz, Spaander, Verloop) to start on the preparatory work. A symposium on standardization ploblems was held at the European Congress of Haematology in Lisbon at the end of which a European Standardizing Committee was founded. The enthusiasm with which the work of this Committee was met was by no means limited to Europe. In order to be able to continue this task on a world wide level, the Managing Board of the Committee was enlarged by members from other Continents on the occasion of yet another Standardizing Symposium which took place during the International Congress of Haematology in Stockholm. The committee was given the name of International Committee for Standardization in Haematology (ICSH). The statutes were drafted and subsequently endorsed at the European Congress of Haematology in Strasbourg in 1965. All the proceedings of the standardizing symposia held up to date are published in the transactions of the ICSH: Erythrocytometric Methods and their Standardization [Bibl. haemat. *18* (Karger, Basel/New York 1964)]. Standardization

* Paper held at the 2nd Latin-American Symposium on Standardization in Mar del Plata on December 11th, 1965. My journey was supported by the Deutsche Forschungsgemeinschaft.

Documentation and Normal Values in Haematology [Bibl. haemat. *21* (Karger Basel/New York 1965)] Standardization in Haematology III [Bibl. haemat. *24* (Karger Basel/New York 1966)]

The lively interest with which the standardizing symposia and the above mentioned publications were followed shows that the initiative was indeed justified nevertheless, there are bound to be many who ask themselves why it is necessary to standardize haematology on an international level, who should undertake this task, how and where it should be done and what exactly should be standardized. The object of this paper is an endeavour to answer briefly these questions.

Standardization in haematology is a necessity to the well-being of our patients and to the scientific work in this field. This can best be illustrated by the following fictive example When Miss SMITH boarded the plane in London, she was still in perfect health, her blood picture normal, with a haemoglobin level of about 80 %. On arriving in Stockholm two hours later her haemoglobin had sunk to a value of barely 60 %! It was fortunate that no over zealous colleague got hold of these values, as he would have sent her straight to hospital to have a transfusion, in spite of the fact that the young lady was just as fit as when boarding the plane. The bloodloss was not accounted for by an error of measurement, but by diverging opinions in the two countries on the millimolar extinction coefficient on the one hand and on the normal value of haemoglobin on the other This incident naturally never really occurred and such a situation could no longer arise nowadays, as at least in Europe haemoglobinometry has been standardized. However serious misunderstandings could arise, for instance, in treatment with anticoagulants when patients travel abroad and many other examples could be cited. It is becoming more and more common for physicians to have to treat patients form some far away corner of the earth. These patients often bring medical reports with them, to which one adds one s own report, and it is evident that these are quite useless to the patient if they are not co-ordinated to have the same values.

Not only the patient s health but also research work can be impaired if misunderstandings arise between scientists. A particularly striking example thereof was the violent disagreement which took place more than 50 years ago between two leading heamatologists, PAPPENHEIM AND TÜRK, with the result that the planned *First International* Congress of Haematology was delayed by a quarter of a

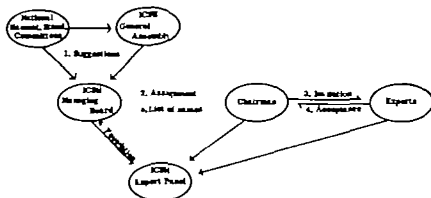


Fig 1 The foundation of expert panels.

century! Yet this quarrel, as the two opponents realized themselves later on, arose chiefly on account of discrepancies in the terminology and was nothing more than a misunderstanding However even today situations frequently arise in which haematologists are at cross purposes, only because the haematological nomenclature is so divergent.

Who is to straighten out this state of affairs? Naturally those specialists who are most familiar with the problems in question It goes without saying that is is necessary to call upon the services of many different scientists to solve such diverse problems. In order to be able to give a correct answer to the many questions which arise, the ICSH sets up an Expert Panel for each separate problem. Scientists from all five continents are invited to attend. A list of the participating specialists is published, and, if considered necessary further participants are included. The members of the Expert Panel first check the pertinent literature, discuss possible solutions, hold symposia and finally draft a proposal. This proposal is then submitted to the Managing Board of the ICSH, published and subsequently approved by the General Assembly of the ICSH. By this procedure access is gained to valuable criticism and suggestions from all parts of the world, so that eventually an international regulation can be worked out which is in every respect satisfactory (Fig 1 and 2)

These international regulations are not only intended for the limited circle of those haematologists who are interested in problems of standardization and who take part in the standardizing work, on the contrary they should reach every general practitioner through-

Fig. 2. The elaboration of standards.

out the world. Only then does standardization on an international level make sense. With this in mind, the international standardizing committee has encouraged from the very start the foundation of national and regional standardizing committees throughout the world, whose task it is to publish and promote locally the international resolutions and to see to it that they are put into practice. The best of resolutions would become meaningless if, for instance, it were not adopted by the South American industry for the simple reason that they were not informed thereof. We know from personal experience that it is almost impossible nowadays to keep up with all the literature, even in the narrowest of fields. It is, therefore, too much to expect every general practitioner and every manufacturer of haematological apparatus to be familiar with a regulation which was published in some foreign book. An Argentinian committee, however will certainly know in which Argentinian publications a revised translation of this resolution should appear and in addition which manufacturers should be notified personally. A national committee will also be in a position to give information on apparatus and preparations in its own country.

How should such a national or regional standardizing committee be established? It is of paramount interest to give this problem very careful thought, as the outcome of well intended work could be thwarted by an inadequate committee or by omitting to consult important authorities. In the first instance such competent autho-

rities as the Ministry of Health and Welfare and the national re presentative of the International Standard Organization (ISO) must be consulted, furthermore the societies of haematology medicine, pediatrics, clinical pathology etc. must be referred to Finally personal advice is to be sought from prominent haematologists throughout the country Once the competent authorities, societies and leading specialists have approved the formation of a national Standardizing Committee and promote its activities, a successful outcome can be expected, as was already the case in many countries. It is, of course, essential that those scientists of whom the committee is made up should not only possess personal initiative and a talent for organization, but also the necessary technical and professional knowledge. Members of the committee should therefore not be limited to specialists connected with such branches of haematology as morphology immunhaematology coagulation etc., but should include chemists, physicists and statisticians (Fig 3)

Such national committees already exist in many countries through out the world and are doing valuable work. South America will undoubtably follow suit. There are haematological societies in many Latin American countries who have achieved a great deal over the past decades. Whether each country should found its own inter national standardizing committee or whether for instance, several Central American countries should join forces in forming a regional committee is a question which is impossible for a European haematologist to answer It would be desirable, therefore, for the national committees within one continent to keep in touch with one another thus enabling mutual assistance. Experience has proved it to be ad vantageous for national or regional committees to exist as independent corporations. For this reason in the Germany "Haemometer Test Board Institute for Standardization and Documentation in Haematology (Hämometerprüfstelle, Institut für Standardisierung und Dokumentation in der Hämatologie) was founded as a legally registered association. I will be happy to supply any persons inter ested with the statures (in German) of this society

The national committees are members of the ICSH and natur ally collaborate with the managing Board of the ICSH not only by promoting the Board's resolutions, but also by informing the Board of current trends, by indicating the degree or urgency of the various pending questions, by suggesting suitable persons to solve them and by expressing their views on preliminary proposals.

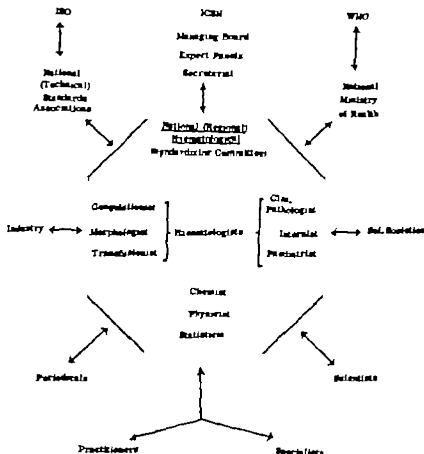


Fig. 3. The proposed composition of national standardizing committees.

Table I
The expert panels of the ICSH.

Topic	Chairman
Blood Cell Counts	J. W. Stewart (London)
Coagulation Testing Procedures	R. J. Ellers (Kansas City)
Cytochemistry in Haematology	B. Thorell (Stockholm)
Determination of Packed Cell Volume	W. H. Crosby (Boston/Mass.)
Erythrocyte Sedimentation Test	G. A. Alexeieff (Moscow)
Evaluation of Normal Values in Haematology	S. Watanabe (Hiroshima)
Haematinics	G. Izak (Jerusalem)
Haematological Documentation	G. Astaldi (Tortona)
Haematological Serology	J. Crookston (Toronto/Ont.)
Haematological Terminology	L. Heilmeyer (Freiburg/Br.)
Haemoglobinometry	J. Spaander (Utrecht)
Red-Cell Fragility	S. M. Lewis (London)

The final question is what should be standardized. The answer to this query is simple: all haematological methods, haematological terminology and the documentation of haematological findings and literature. It is, of course, impossible to resolve all these problems at once. A host of scientific questions have to be gone into very thoroughly before a method can be standardized. Basic methods should obviously be given priority: those of lesser importance can be attended to at a later date. For these reasons the ICSH started by standardizing haemoglobinometry and has achieved thereby excellent results. For the immediate future the ICSH is planning studies on roughly a dozen topics and has formed Expert Panels for this purpose (Table I).

Summary

The standardization of haematological methods, nomenclature and documentation is of primary importance. This work is currled out by the International Committee for Standardization in Haematology (ICSH). The work of the ICSH is prepared by Expert Panels and carried out by national or regional committees. The author expounds his personal opinion on how this task can best be performed.

Résumé

La standartisation de la nomenclature, de la documentation et des méthodes hématologiques est d'une importance primordiale. Ce travail est accompli par le Comité International pour la Standartisation en Hématologie (CISH).

Le travail du CISH est préparé par des commissions d'experts et exécuté par des comités nationaux ou régionaux. L'auteur donne son opinion sur la façon dont cette tâche peut être le mieux accomplie.

Zusammenfassung

Die Standardisierung hämatologischer Methoden, der Terminologie und der Dokumentation sind dringend notwendig. Diese Arbeit wird vom «International Committee for Standardization in Haematology (ICSH)» durchgeführt. Die Arbeit des ICSH wird von «Expert Panels» vorbereitet und von nationalen, bzw. regionalen Standardisierungskomitees durchgeführt. Der Autor legt seine persönliche Auffassung dann dar, wie seiner Meinung nach diese Arbeit am vorteilhaftesten geleistet werden kann.

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From the Cancer Research Unit, Veterans Admin. Hospital, Bronx, New York

The Rauscher Virus: A Mixture of the Friend Virus and of the Mouse Leukemia Virus (Gross)?

LUDWIK GROSS

A leukemogenic virus isolated a few years ago by RAUSCHER (12) has been employed extensively in studies dealing with experimental mouse leukemia. This virus has two principal pathogenic properties: it can induce in susceptible mice (a) the characteristic syndrome of FRIEND disease, or in mice of certain strains, and in rats, it can also induce (b) lymphoid leukemia, including thymic and generalized lymphosarcomas.

Development of Friend Syndrome in Mice Following Inoculation of the Rauscher Virus

When inoculated into either suckling or young adult (3 to 4 weeks old) mice of susceptible strains, such as C3H, BALB/c, or Swiss, the RAUSCHER virus induces, after a short latency of only a few weeks, a characteristic syndrome indistinguishable from that induced with the FRIEND virus (1, 9). Considerable enlargement of spleen, and liver represent the most striking macroscopic feature of the induced disease. Characteristically, and within a relatively short time, the spleen may become enormously enlarged, and filled with fragments of destroyed cells, and altered blood (Fig. 1). There are no thymic tumors, and the peripheral lymph nodes are not enlarged. This is in striking contrast to the great majority of the conventional forms of mouse leukemia (8). The white blood cell count is considerably increased, and the peripheral blood smears show evidence of pronounced anemia, presence of many nucleated red cells, and characteristically also very large numbers of smudge cells, among a variety of cells of the myeloid series in different

Aided, in part, by grants from the Damon Runyon Memorial Fund and the American Cancer Society



Fig. 1. Characteristic enlargement of spleen and liver induced with the RAUSCHER virus. This C3H(f) female mouse was inoculated (0.3 ml i. p.) when less than 5 weeks old, with 5 RAUSCHER virus filtrate, and was sacrificed 25 days after inoculation. Note the very large spleen and also enlarged liver. Significantly, the thymus is not enlarged, and there is no enlargement of peripheral lymph nodes. WBC 158,200 mm³ with 347 smudge cells per 100 WBC, and presence of many nucleated red cells, among variety of white cells predominantly of the myeloid series in peripheral blood. Hb 7.2 gm/100 ml. This macroscopic and microscopic pathologic picture was indistinguishable from that induced with the FRIEND virus (1, 9)

phases of maturation. On microscopic examination of liver and spleen, there is considerable infiltration with abnormal white cells, with areas of destruction and necrosis; some of the other organs may also show infiltration with abnormal cells, but to a lesser extent. In most instances the disease is progressing rapidly and the animals



Fig. — Thymic lymphosarcoma induced in rat with the RAUSCHER virus. The RAUSCHER virus, after 10 consecutive passages through rats, was inoculated (0.4 ml of 10^4 filtrate p.) into suckling less than 3 days old, Sprague Dawley female rat. After latency of 2 months, this rat developed large thymic lymphosarcoma, and multiple lymphosarcomas in the spleen, liver cervical and mesenteric lymph nodes. WBC 58,500 mm^3 with 10^4 lymphoblasts among predominantly lymphocytes in peripheral blood. Hb 9 gm 100 ml. This pathologic picture of lymphoid leukemia was essentially identical with that usually resulting from inoculation of the mouse leukemia virus (GROSS).

die within 2 or 3 months after inoculation. This macroscopic, and microscopic picture of the disease induced in mice with the RAUSCHER virus, is identical with that resulting from inoculation of the FRIEND virus (1 9)

The current difficulties in clarification and classification of the mouse leukemia viruses (8, 9) have been increased by the introduction into the study of experimental mouse leukemia of a progressive disease of the hematopoietic system developing in mice following inoculation of the FRIEND virus (1). This curious syndrome seems to be a true "laboratory disease" and appears to be exceedingly rare under natural life conditions, if it occurs spontaneously at all. This unusual disease of the hematopoietic system probably belongs to the broad group of leukemias, although it is very different from any of the usual forms of leukemia occurring in mice spontaneously or induced by radiation, chemicals, or hormones.

Development of Thymic Lymphosarcomas and Other Conventional Forms of Mouse Leukemia in Mice and Rats Following Inoculation of the Rauscher Virus

Under certain experimental conditions, inoculation of the RAUSCHER virus filtrate may induce lymphoid leukemia with the characteristic development of thymic and generalized lymphosarcomas. This may take place when suckling or weanling (3 to 4 weeks old) mice of certain strains, such as C57 Black or C57 Brown relatively resistant to the FRIEND virus, or when suckling rats, are inoculated with the RAUSCHER virus filtrate (12, 9).

The natural resistance of mice of certain strains, such as C57 Brown, to the induction of FRIEND disease, following inoculation of the RAUSCHER virus, is only relative, and is apparent when weanling mice are employed for bio-assay. On the other hand when newborn, or suckling, C57 Brown mice are inoculated with the RAUSCHER virus filtrate, some will develop after a short incubation period acute enlargement of spleen and liver with the characteristic blood picture of FRIEND syndrome; other mice in such group will survive, but develop after a prolonged latency lymphoid leukemia with thymic and generalized lymphosarcomas; a few develop a "mixed" form, i.e. thymic and generalized lymphosarcomas with blood morphology similar to that observed in the FRIEND syndrome.

It should be pointed out that similar results could be obtained employing the FRIEND virus filtrate for inoculation of suckling, and 3 to 4 weeks old mice (9). Furthermore, the FRIEND virus can also induce thymic lymphosarcomas, as well as lymphatic, stem-cell, or myeloid leukemia, following inoculation into suckling rats (11, 9). This pathogenic action of the FRIEND virus may be due to the presence in the FRIEND virus filtrate of the GROSS mouse leukemia virus component (9).

The interesting ability of the RAUSCHER virus to induce (a) the hepato-splenomegaly syndrome of FRIEND disease, with its characteristic pathologic features, and striking blood picture, or (b) conventional form of mouse leukemia, frequently associated with the development of a thymic lymphosarcoma, such as lymphatic, stem cell, or myeloid leukemia, could be explained by an assumption that a single virus, present in the RAUSCHER filtrate, has a wide range of pathogenic potential, and is capable of inducing both, the FRIEND syndrome and one of the conventional forms of mouse leukemia, such as thymic lymphosarcoma.

On the other hand, it would be also possible to assume that what is now considered to be the RAUSCHER virus may actually represent a mixture of two distinct viruses, the FRIEND virus, and in addition the GROSS mouse leukemia virus (8, 9).

A Mixture of Friend Virus and of Mouse Leukemia Virus (Gross)?

Let us consider the possibility that the RAUSCHER virus strain, and actually though to a lesser extent, also the FRIEND virus filtrate (9) consists of a mixture of the FRIEND virus, in a relatively high titer and of the GROSS mouse leukemia virus (*i. e.* the passage A virus) (4) the latter in a relatively lower concentration. Following inoculation of such a filtrate into animals susceptible to both viruses, either form of disease could be induced. However the FRIEND virus induces disease after a relatively shorter latency and for that reason, when susceptible mice are inoculated they usually develop and die from, the FRIEND syndrome before they have an opportunity to develop lymphatic leukemia, or another form of leukemia caused by the mouse leukemia virus (9) the latter requires a considerably longer latency period for induction of disease (2, 4 6).

On the other hand, if the RAUSCHER filtrate, presumably consisting of a mixture of the two viruses, is inoculated into mice of certain inbred strains such as C57 Black of C57 Brown, or into suckling rats, which are relatively resistant to the induction of the FRIEND syndrome, one of the conventional forms of mouse leukemia such as lymphatic, or stem-cell leukemia, may develop as a result of the pathogenic action of the GROSS mouse leukemia virus present in the filtrate. Characteristically most of these animals develop thymic lymphosarcomas, as a rule absent in mice with FRIEND virus-induced syndrome.

In some instances, "mixed form" representing mixture of both the FRIEND disease and one of the conventional forms of mouse leukemia, such as thymic lymphosarcomas, can also be induced in the same host. Pathologic manifestations which could be attributed to both viruses have been observed quite frequently in our laboratory in some of the mice, in certain instances also in rats, inoculated with the RAUSCHER virus strain, and occasionally also following inoculation of the FRIEND virus filtrate. The combined effect of both virus components may in some instances result in the induction, particularly in rats, of acute myelogenous leukemia with considerably enlarged spleen, very advanced anemia, presence of nucleated red cells, and characteristic smudge cells in peripheral blood, among variety of white cells of the myeloid series. Whether the possibility of synergistic action of both viruses should be considered remains an open question, since the mouse leukemia virus (GROSS) alone may also induce in certain instances (5, 6) particularly in rats (7) acute myelogenous leukemia. Furthermore, the FRIEND virus is also

capable of inducing an acute syndrome with blood morphology which could be classified as disease belonging to the group of erythroid or myelogenous leukemias, even though there may exist some reservations as to the true nature of this curious disease.

Results of experiments carried out recently in our laboratory are consistent with the assumption that the RAUSCHER virus strain actually consists of a mixture of two viruses.

Attempt to Inhibit the Friend Virus Component of the Rauscher Virus Filtrate by Serum Neutralization

An attempt was made to neutralize *in vitro* the FRIEND virus component, presumably present in the RAUSCHER virus filtrate, by specific immune serum. The RAUSCHER virus was mixed with immune serum obtained from rabbits that had received several injections of FRIEND virus filtrates. The mixture was then inoculated into suckling, and also into 3 weeks old C3H(f) mice. After neutralization with anti-Friend serum, the RAUSCHER virus strain filtrate was still able to induce lymphatic leukemia; however its potency to induce FRIEND disease was considerably diminished (9)

These experiments are now repeated with an effort to follow more quantitative approach to the neutralization test; it is hoped that under more precise experimental conditions it will be possible to inhibit completely the FRIEND virus component of the RAUSCHER virus filtrate.

Attempt to Eliminate from the Rauscher Virus Filtrate the Friend Virus Component by Serial Passage Through Rats

Inoculation of either the FRIEND or the RAUSCHER virus, induces in rats, as a rule, lymphatic leukemia, in some instances stem-cell leukemia, and occasionally also the myelogenous form. Characteristically thymic lymphosarcomas develop in the great majority of such animals (Fig 2)

Since rats do not develop typical FRIEND disease, as seen in mice but react, following inoculation of either FRIEND or RAUSCHER virus by the development of a conventional form of leukemia, it appeared of interest to pass the RAUSCHER virus serially through rats* in an attempt to determine whether such rat adapted RAUSCHER virus would still retain its ability to induce typical Friend disease when inoculated back into mice.

Accordingly the RAUSCHER virus, harvested from mice, was inoculated in form of 5 / filtrates, prepared in the usual manner

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* The virus employed in our studies was received from Dr F J RAUSCHER, National Cancer Institute, in 1962, and has been maintained since that time in our laboratory in mouse-to-mouse passages The method of preparation of filtrates was described previously (9)

employed in our laboratory (4 6) into suckling, less than 6 days old, Sprague Dawley rats. As soon as the inoculated rats developed leukemia, they were sacrificed the virus was harvested from the leukemic rat donors, and inoculated again into newborn rats. This procedure was repeated serially and the RAUSCHER virus was passed through 10 consecutive rat to-rat passages, inducing in practically all inoculated animals thymic tumors and disseminated lymphosarcomas, occasionally stem-cell, or myelogenous leukemia, very similar to forms induced with the passage A virus (GROSS) From each consecutive rat to-rat passage, an attempt was made to inoculate the rat adapted virus back into suckling, or 3 to 4 weeks old, C3H(f) mice, known to be uniformly susceptible to the induction of FRIEND disease

After the initial 2 or 3 consecutive rat to-rat passages, the RAUSCHER virus lost much of its ability to induce a typical FRIEND disease following inoculation into either suckling or 3 weeks old C3H(f) mice, the rat-adapted virus induced either typical lymphatic leukemia, or a mixed form, manifested by the development of thymic lymphosarcomas and also some of the features such as a very large spleen or the characteristic blood picture, of the FRIEND disease only exceptionally however did such a virus induce a typical FRIEND syndrome (Table I) After 7 to 8 consecutive rat

Table I

Results of inoculation of RAUSCHER virus strain into suckling and weanling C3H(f) mice, following 1 to 10 consecutive passages through rats.

No. of consecutive passages through rats	Average age at inoculation days	No of mice inoculated	Form of leukemia induced			Average age leukemia dev months	No. of negative mice	Average age negative mice, months
			A	Type F*	mixed			
1 to 3	28	29	8		3	< 4	18	8
	3	16	7	2	7	< 3		—
4 to 6	32	47	5		1	< 8	41	12
	5	14	6	1	7	< 3		—
7 to 8	35	30	1			9	29	10
	5	2	2			< 3		—
9 to 10	26	30	1			< 4	29	5—8*
	5	7	7			< 3		

Type A, usually lymphatic or stem-cell, with thymic tumors, enlargement of peripheral lymph nodes, etc., similar to that induced with passage A virus

Type F (FRIEND) hepato-splenomegaly with no thymic tumors, and no peripheral lymph node enlargement. Characteristic blood morphology showing presence of variety of white cells of the myeloid series, large number of 'smudge cells' and many nucleated red cells also severe anemia.

These mice are still alive, and under observation, t ages varying from 5 to 9 months.

to-rat passages, however the RAUSCHER virus lost its characteristic potency to induce symptoms of FRIEND disease in susceptible mice, but retained nevertheless its ability to induce lymphatic leukemia in either mice or rats. Thus, when after 7 to 10 rat-to-rat passages, the RAUSCHER virus, harvested from leukemic rat donors, was inoculated into suckling or 4 to 5 weeks old C3H(f) mice none developed either the characteristic acute enlargement of spleen and liver or the distinctive blood picture typical for the FRIEND disease (Table I) It appeared therefore, that after several consecutive rat-to-rat passages, the RAUSCHER virus lost its FRIEND virus component. The same rat-adapted virus inoculated into 9 suckling mice induced in all of them thymic and generalized lymphosarcomas (Table I) presumably caused by the mouse leukemia virus (GROSS) component remaining in the filtrate. Only 1 out of 30 mice in each of 2 consecutive experiments, inoculated at 4 to 5 weeks of age, developed lymphoid leukemia, during the observation time thus far elapsed (Table I) mice of that age are less susceptible than suckling animals to the mouse leukemia virus (GROSS) (2 4 6)

The leukemogenic potency of the rat-adapted RAUSCHER virus for suckling rats, inducing predominantly thymic lymphosarcomas, became slightly increased for that species after consecutive rat to-rat passages as evidenced by increased incidence of induced lymphomas, and shorter latency*

Results of Serial Rat-to-Rat Passage of the Mouse Leukemia Virus (Gross)

As comparison, it is of interest to stress at this point that serial passage through rats of the passage A mouse leukemia virus did not alter its pathogenic potential determining the form of induced disease. Thus, the mouse leukemia virus (GROSS) isolated originally from spontaneous mouse leukemia (4) was found to be pathogenic not only for mice, but also for rats (7) inducing in that species forms of leukemia and lymphomas essentially similar to those induced in mice The virus has now been passed serially through 21 consecutive rat-to-rat passages, inducing predominantly thymic and disseminated lymphosarcomas, but also lymphatic, stem-cell, and myelogenous leukemia.

At several passage intervals, after 8, 10, 13 17 18, 20, etc consecutive rat-to-rat passages, the mouse leukemia virus, harvested from leukemic rats, was inoculated back into suckling C3H(f) mice, inducing the same forms of leukemia and lymphomas as did the original virus that had been harvested from leukemic mice (10). Thus serial passage through rats might have increased the titer of the virus for the rat species

* Incidence of leukemia induced in rats practically 100% average latency approximately 3 months (GROSS, L Unpublished experiments) In this respect, therefore the rat-adapted RAUSCHER virus is indistinguishable from the passage A (GROSS) leukemia virus passed in rats (7)

(7 10) but did not alter the intrinsic pathogenic property of the virus, expressed by the form of leukemias and lymphomas induced on inoculation tests in the host of origin, i. in mice.

Experiments are now in progress in our laboratory dealing with serial rat-to-rat passage of the Friend virus. Thus far only 3 consecutive rat-to-rat passages have been performed, and the inoculated rats developed large thymic lymphosarcomas. The rat adapted Friend virus, harvested from leukemic rats, after 2 consecutive passages in that species, was inoculated back into 3 to 4 weeks old C3H(f) mice out of 20 inoculated mice 4 developed typical lymphatic leukemia the remaining 16 mice are thus far in good health, 6 months after inoculation none developed the Friend disease. The original Friend virus, carried in our laboratory in mouse-to-mouse passage induces 100% incidence of the characteristic Friend syndrome following inoculation into less than 4 weeks old C3H(f) mice

Further rat-to-rat passages of Friend virus will be carried out however results of these preliminary experiments suggest that the Friend virus could be eliminated from Friend virus filtrate by serial passage through rats, leaving the mouse leukemia virus (Gross) component.

Neutralization in vitro of the Lymphatic Leukemia Virus Component of the Rauscher Virus Filtrate with Immune Mouse Leukemia (Passage A—Gross) Virus Serum

Since the rat-adapted Rauscher virus strain retained its potency to induce in rats thymic and generalized lymphosarcomas, an attempt was made to determine whether this pathogenic potency of the Rauscher virus strain could be inhibited by an immune serum obtained from rabbits immunized with the passage A virus (Gross) The Rauscher virus strain, that had been passed through 8 consecutive rat to-rat passages, was employed The virus filtrate was prepared from thymic tumors and spleens of leukemic rat donors, employing our method used routinely (4 6, 10) for the preparation of passage A filtrates. A 10^{-3} dilution of the rat adapted Rauscher virus strain filtrate was then mixed *in vitro* with an equal amount of undiluted immune serum, obtained from rabbits that had received several consecutive injections of passage A virus harvested from leukemic C3H(f) mice with Freund adjuvant (10) In a simultaneous control experiment a 10^{-3} dilution of the virus filtrate was mixed with equal amount of undiluted serum obtained from rabbits that had received several injections of normal C3H(f) mouse organs, with Freund adjuvant (10) All sera were inactivated at 56°C for 1 h prior to neutralization tests. In a third group as an additional control, the 10^{-3} virus filtrate dilution was mixed with an

Table II

Neutralization in vivo of lymphatic leukemia component of the RAUSCHER virus strain with immune passage A virus serum.

Antigen employed for preparation of rabbit serum	Serum dilution	Virus fil. dilution **	No. of rats inoculated	No. dev. leukemia	Average age leukemia dev. mos.
Passage A virus harvested from mice (with Adj.)	undiluted	10^{-4}	5	0	—
Normal mouse organs (with Adj.)	undiluted	10^{-8}	6	6	< 5
Saline controls		10	6	6	< 4

Rat-adapted" following 8 rat-to-rat passages.

Virus filtrate incubated with serum at room temperature for 30 min, then at +4 °C for 3½ h. Method of preparation of serum was previously described (10)

** Suckling < 7 days old Sprague Dawley rats inoculated 0.6–0.8 ml i. p.

Lymphatic leukemia with thymic tumors and generalized lymphosarcomas. (Rats in the first group, which remained in good health, free from leukemia, were observed for 10 months)

equal amount of physiological saline solution. The virus-serum mixtures, as well as the virus-saline control, were incubated at room temperature for 30 min, followed by incubation at +4 C for 3½ h, and were then inoculated into suckling Sprague Dawley rats. The results, summarized in Table II indicate clearly that the passage A immune rabbit serum inhibited completely the ability of the RAUSCHER virus strain to induce lymphatic leukemia, following inoculation into suckling rats. There was no inhibiting effect whatever of the normal mouse organs serum. Virus filtrate mixed with normal mouse organs serum, as well as the virus filtrate diluted only as a control, with equal amount of physiological saline solution induced in all inoculated rats lymphatic leukemia with characteristic large thymic lymphosarcomas, and also generalized lymphosarcomas in spleens, livers, and other organs.

Mixture of Two Oncogenic Viruses in One Filtrate A Common Difficulty in Experimental Mouse Leukemia Studies

It would not be the first time to observe that a filtrate prepared from leukemic mouse tissues may contain a mixture of two distinct oncogenic viruses. It may be of interest to recall at this point

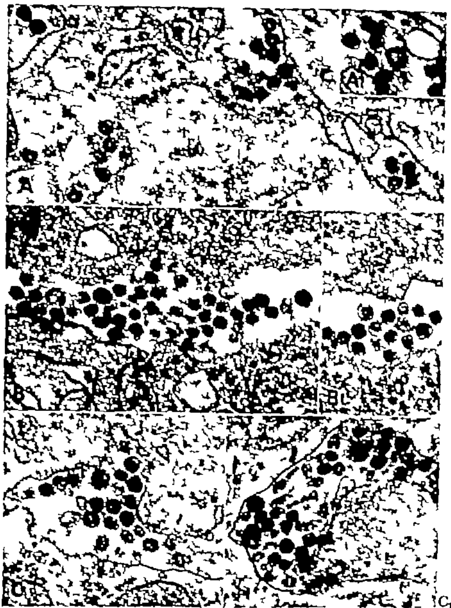


Fig. 3. Electron micrographs of ( ) RAUSCHER virus, (b) FRIEND virus, and ( ) mouse leukemia virus (GROSS). Electron microscopic studies of ultra-thin sections of leukemic organs, such as spleen, lymph nodes, etc. of mice and rats, that developed disease following inoculation of either RAUSCHER, FRIEND, or GROSS virus, revealed presence of spherical particles averaging about 100 mμ in diameter essentially identical in their morphology and location, either of the doughnut-type or with electron-dense, centrally located, nucleoids. They were found either in intercellular spaces, within cytoplasmic vacuoles, or budding, usually from the edges of the cytoplasmic cell membranes of variety of cells. *Upper Section* (A and A_1) Fragments of spleen from C3H(f) mouse with

the early studies on mouse leukemia. In the initial experiments (2) when the mouse leukemia virus was first isolated from organs of Ak mice with spontaneous leukemia, some of the extracts prepared from such organs inoculated into newborn C3H mice induced parotid gland tumors, instead of leukemia (3) A few animals developed both, leukemia and parotid tumors. The possibility had to be considered that a single virus was responsible for the induction of either leukemia, or parotid gland tumors. It was soon realized, however that extracts prepared from leukemic mouse tissues contained two different viruses, one causing leukemia, and another causing parotid, and other salivary gland, tumors (3) The mouse leukemia virus, and the parotid tumor virus, even though initially present simultanously in the same filtrates, could readily be separated by ultracentrifugation, or by differential heating since the mouse leukemia virus was found to be larger and more susceptible to heat. In general, however the separation of two different viruses present in the same filtrate may be difficult, particularly if such viruses have similar physical, and biological properties.

Summary

The Rauscher virus can induce in susceptible mice considerable enlargement of spleen and liver and characteristic blood picture pathologic features identical with those resulting from inoculation of the Friend virus. In certain strains of mice however and also in rats, the Rauscher virus can induce thymic lymphosarcomas, and other conventional forms of leukemia commonly observed following inoculation of the mouse leukemia virus (Gross) This dual pathogenic potency could be explained by an assumption that the Rauscher virus filtrate consists of mixture of the Friend virus, and of the mouse leukemia virus (Gross) Results of experiments here reported are consistent with such an assumption, Following several serial passages through rats, species relatively resistant to the Friend virus, the Rauscher virus lost its ability to induce Friend virus syndrome on mouse bio-assay Furthermore, the ability of the rat-adapted Rauscher virus to induce thymic lymphosarcomas could be inhibited by neutralization *in vitro* with the Gross mouse leukemia virus immune serum.

The mouse leukemia virus (Gross) as well as the Friend virus, and the Rauscher virus strain, have similar physical properties, and are indistinguishable in their morphology on electron microscopic examination (Fig. 3).

Rauscher virus-induced Friend syndrome (37 450). *Middle Section* (B and B_1) Fragments of spleen of C3H(f) mouse with Friend virus-induced disease (33,600) *Lower Section* (C) Fragment of spleen from Sprague Dawley ra with passage A (Gross) irus-induced myeloid leukemia (37 450). (C_1) Fragment of lymph node from C3H(f) female mouse with lymphoid leukemia induced with passage A virus (33600) (Electron micrographs prepared in our laboratory by Dr D G Feldman in cooperation with the author)

Résumé

Le virus de Rauscher peut provoquer chez des souris susceptibles un agrandissement considérable de la rate et du foie, et une formule sanguine caractéristique: traits pathologiques identiques avec ceux résultant d'une inoculation du virus de Friend. Dans certaines souches de souris par contre, ainsi que chez le rat, le virus de Rauscher peut provoquer des lymphosarcomes thymiques et d'autres formes conventionnelles de leucémies communément observées après l'inoculation du virus de leucémies de la souris (Gross). Ce double pouvoir pathogène serait peut-être dû à ce que le filtrat du virus de Rauscher consisterait en un mélange du virus de Friend et du virus de la leucémie de la souris (Gross). Les résultats des expériences rapportées ici concordent bien avec cette hypothèse. Après plusieurs passages chez le rat, une espèce relativement résistante au virus de Friend, le virus de Rauscher perd sa faculté d'induire le syndrome de Friend. En plus, le pouvoir pathogène du virus de Rauscher adapté au rat, de produire un lymphosarcome du thymus, peut être neutralisé *in vitro* par un sérum spécifique préparé à l'aide du virus de Gross.

Zusammenfassung

Das Rauscher-Virus kann bei empfindlichen Mäusen eine beträchtliche Vergrößerung von Milz und Leber hervorrufen, pathologische Veränderungen, die mit denjenigen nach Inokulation des Friend-Virus identisch sind. Bei gewissen Mäusestämmen und auch bei Ratten kann das Rauscher-Virus Thymus-Lymphosarkome und andere gewöhnliche Leukämieformen erzeugen, wie sie nach Inokulation des Mäuseleukämie-Virus (Gross) beobachtet werden. Diese zweifache pathogene Eigenschaft könnte mit der Annahme erklärt werden, daß das Filtrat des Rauscher-Virus aus einem Gemisch des Friend-Virus und des Mäuseleukämie-Virus (Gross) besteht. Die Ergebnisse der hier mitgeteilten Versuche stehen mit dieser Annahme in Übereinstimmung. Nach mehreren Passageserien bei Ratten, einer gegenüber dem Friend-Virus relativ resistenten Species, verliert das Rauscher-Virus seine Fähigkeit, das Friend-Virus-Syndrom im Mäuseversuch zu erzeugen. Ferner konnte die Fähigkeit des an Ratten adaptierten Rauscher-Virus zur Erzeugung von Thymus-Lymphosarkomen gehemmt werden durch Neutralisation *in vitro* mit dem Immunserum der Mäuseleukämie von Gross.

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Estimation of Foetal Haemoglobin in Leukaemia

G. Bartolozzi and L. Mariani

The concentration of foetal haemoglobin (Hb F) in children after the first year of life, and in adults is usually not greater than 1–2 % of the total haemoglobin (1–4). Levels higher than this in patients older than one year suggests in the first place a rare congenital anomaly known as persistence of foetal haemoglobin (5, 6). It is pathognomic of this condition that the foetal haemoglobin is distributed evenly in all the red cells, so that the percentage of foetal haemoglobin in each cell is similar to that in the total population of cells.

A raised foetal haemoglobin level is almost always found in *certain other haemoglobinopathies (thalassaemia, sickle cell disease)* in which there is a congenital abnormality affecting the synthesis of the β-chains. In such conditions the foetal haemoglobin is distributed irregularly in the red cells, so that one cell may have an abnormally high concentration while others are completely free from foetal haemoglobin (3). Investigations of the foetal haemoglobin levels in other haematological disorders have shown the levels to be significantly raised above normal in numerous pathological conditions: *megaloblastic anaemia (1, 7, 8), aplastic an*aemia (1, 7–11) and leukemia (1, 7, 10, 12–20). From this point of view we have investigated the levels of foetal haemoglobin in children with leukaemia of various types, and at various stages of the disease.

Material and Method

We studied 22 cases of leukaemia in children aged 13 months to 12 years, of whom 16 had acute leukaemia, 3 had chronic myeloid leukaemia, and 3 had acute myeloid leukaemia; all were admitted to the paediatric clinic in Florence during the last years.

FETAL HAEMOGLOBIN %

L. A. = acute lymphocytic leukaemia
M. Cr = chronic granulocytic leukemia
M. A = acute granulocytic leukaemia.

The foetal haemoglobin levels of these patients was measured one or more times during the course of their illness, by means of Betke's method (14).

The results are set out according to the stage of the disease and the corresponding numbers of red and white cells are also given.

Results

Fig 1 shows the percentages of foetal haemoglobin in our 22 cases Of the 16 children with acute leukaemia 5 had a foetal haemoglobin level of less than 1 %, and the rest varied from 3 to 20 % In cases 1 and 2 in which the test was carried out a second time after an interval of 1 month the initial level was less than 1 % but at the end of the month, the levels of foetal haemoglobin had risen to 4 and 10 % respectively

In all the 6 cases of myeloid leukaemia, acute and chronic, the foetal haemoglobin levels were much higher than normal, varying from 5 to 38.5 % In two of the cases (19 and 21) the levels were the highest found in the whole study being 35 and 38.5 % respectively In case 21 in which the test was repeated three times at intervals of 15 days and 2 months, the foetal haemoglobin level was permanently raised varying from 20 to 38.5 % To exclude thalassaemia, we measured the level of haemoglobin A_2 in the parents of the child, and these were within normal limits.

In none of the patients did we find any positive correlation between the numbers of red cells and the percentage of foetal haemoglobin. It is possible, however that frequent blood transfusion masked a possible relation between these two factors. We found no correlation between percentage of foetal haemoglobin and the white cell count

Discussion

The results we have obtained are more or less in agreement with those of other authors. Vecchio (19) made the first study of foetal haemoglobin in two leukaemic patients in 1948 and found concentrations normal for the age of the subjects. Singer *et al* (14 15) later found in two cases of acute leukaemia and one of chronic leukaemia, that the levels of foetal haemoglobin were significantly raised above normal (3 2–4 3 and 2 6 % respectively) Pologa *et al.* (7 20) in 20 cases of various types of leukaemia, found increased levels of foetal haemoglobin in the haemocytoblastic and chronic myeloid leukaemias, which averaged 6 4 and 4 4 % respectively In one case of haemocytoblastic leukaemia the foetal haemoglobin level was 25 %. Beaven *et al* (1 12 13) investigated 33 cases of leukaemia, of which 5 had raised foetal haemoglobin levels, but none were greater than 10 %. In contrast two other children show

ed very high levels, varying between 15 and 30 % over long periods of time. SHUTTER (16) reported a case of a girl of 12 with acute myeloid leukaemia in which the foetal haemoglobin concentration was 20 % right from the beginning. HARDISTY *et al.* (18) reported the cases of 4 children with myeloid leukaemia of the so-called juvenile type, in which the foetal haemoglobin levels varied between 40 and 50 %. RAPER (8) in 3 cases of a total of 6 of acute leukaemia in children, found an increase in the numbers of red cells containing foetal haemoglobin as compared with normal. KIOSSOGLOU *et al.* (10) after investigating 11 cases of leukaemia in children, found increased foetal haemoglobin levels in the majority and concluded that the foetal haemoglobin was not distributed evenly in all the red cells.

The present results, together with those from the literature show that in the course of leukaemia in children a moderate increase in the foetal haemoglobin levels (<10 %) is very common, while the levels much more increased than this value are encountered only rarely.

We looked into those with very high foetal haemoglobin levels in an attempt to find some common factor. The two cases of BEAVEN (21), the 4 cases of HARDISTY (18), the single case of SHUTTER (16) and our two cases were all patients suffering from acute myeloid leukaemia. BEAVEN (21) and HARDISTY (18) considered that these patients were cases of myeloid leukaemia of the juvenile type, which is an atypical form of leukaemia characterised by thrombocytopenia, poor response to treatment, increased foetal haemoglobin levels, and an age-group of 1½–3½ years. This distinguishes it from the adult type, which affects older children. Such subdivisions however seem to us to be an oversimplification when one considers the foetal haemoglobin levels, since the case of SHUTTER and our two cases, although having raised foetal haemoglobin levels, do not satisfy all the criteria in the accepted definition of the juvenile type of myeloid leukaemia.

Various hypotheses have been put forward to explain the increased foetal haemoglobin levels in leukaemia. With the gradual decline in the synthesis of the γ-chains after birth, and after the substitution of β-chain synthesis (change over from Hb F to Hb A_1) during the first year of life, only few clones of erythroblasts maintains its capacity to synthetise the γ-chains, and these are responsible for the 1 % foetal haemoglobin found in adults.

The appearance of high levels of foetal haemoglobin in leukaemic patients, as well as in other haematological disorders, indicates a return to the synthesis of the γ-chain owing to stimulation of these clones by some sort of feedback mechanism (22-23). The irregular distribution of foetal haemoglobin through the red cell population supports this hypothesis. If this mechanism is admitted, then it can be seen that it is more efficient when it operates early in infancy rather than in adulthood (24). It is probable that this mechanism of the control of the synthesis of foetal haemoglobin is not, or is not only, the effect of low oxygen tensions in the tissues, since it has been shown that in patients with congenital cyanotic and acyanotic heart diseases the change to haemoglobin A_1 is not retarded, nor are there increased foetal haemoglobin levels in older age groups (8, 25).

According to BAGLIONI (3) in severely anaemic patients the stem cells differentiate to red cells after a limited number of cell divisions, whereas in normal erythropoiesis a considerable number of divisions is necessary for maturation from the erythroblast stage to mature red cells. After a small number of divisions the red cells contain considerably larger quantities of foetal haemoglobin than the cells resulting from a large number of divisions. This probably is the mechanism responsible for the high levels of foetal haemoglobin found in anaemic patients.

It has been shown, however, that in leukaemia, besides this hypothetical mechanism, there are other factors influencing the synthesis of foetal haemoglobin and the structure of the red cells. These factors presumably bring about a profound change in the bone marrow of leukaemic patients, and may produce ambient cellular conditions in which the synthesis of one chain is easier than the other. In fact, there are numerous reported cases of a change of blood group in leukaemia (26, 27, 28), and of the appearance of Hb H, which is haemoglobin composed of four β-chains (17, 27).

It therefore seems that in leukaemia there is an increase in the level of foetal haemoglobin to a moderate degree, as has been found in various other haemopoietic disorders. Only rarely there are high levels of foetal haemoglobin as in some acute myeloid leukaemias in infants, or on the other hand Hb H as in some leukaemias of the adults.

Summary

The levels of foetal haemoglobin were investigated in 22 children with leukaemia. Of 16 children with acute leukaemia, 5 had level of foetal haemoglobin lower than 1% and the rest varied between 3 and 20% In all 6 cases of myeloid leukaemia, acute and chronic, the foetal haemoglobin levels were much higher than normal, varying from 5 to 38.5% In two of the cases the levels were the highest that we found in the whole study being 35 and 38.5% respectively

Résumé

Le taux d'hémoglobine foetale été déterminé chez 22 enfants atteints de leucémie. 5 parmi 16 enfants yant une leucémie aiguë avaient un taux d'hémoglobine foetale de moins de 1% et les autres un taux variant de 3 à 20% Les taux d'hémoglobine foetale étaient beaucoup plus hauts que normalement, variant de 5 à 38,5% dans 6 cas de leucémie myéloide aiguë et chronique. Deux de ces malades présentèrent les valeurs les plus élevées de toute l'étude, respectivement 35 et 38,5%.

Zusammenfassung

Bei 22 Kindern mit Leukämie wurde der Gehalt an fotalem Hamoglobin bestimmt. Von 16 Kindern mit akuter Leukämie hatten 5 Werte von unter 1% die übrigen zwischen 3 und 20%. Bei allen 6 Fällen von akuter und chronischer myeloischer Leukämie waren die Werte wesentlich höher als normal und schwankten zwischen 5 und 38,5% Zwei dieser Patienten zeigten die höchsten Werte der gesamten Untersuchung, nämlich 35 und 38,5%.

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The Migration of Cells to the Thymus

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It has been shown that lymphoid cells of extra thymic origin can migrate to the thymus under a variety of experimental conditions. When marrow labelled with the T6 chromosome marker is administered to lethally irradiated mice, donor cells appear in the recipients bone marrow and thymus (6) and only later throughout the lymphoid system (17). By contrast, thymic and lymph node lymphocytes appear in lymph nodes and spleen (6, 18) as do thoracic duct lymphocytes (6). Similar results have been reported for unirradiated mice with thymocytes labelled with thymidine (21) and for rats with thoracic duct cells labelled with tritiated adenosine (8). Splenic cells can however migrate to the thymus in irradiated mice (7), possibly since this organ contains some haemopoietic tissue. These findings have led to the proposal that lymphoid cells leave the bone marrow, enter and proliferate in the thymus and then distribute themselves in lymph nodes (6). The observation (1) that bone marrow contains two separate factors, one responsible for marrow regeneration and the other for thymic regeneration in lethally irradiated recipients, supports this conclusion.

Further evidence that lymphocytes reside in the thymus during one stage of their maturation, but do not necessarily originate in this organ is derived from experiments involving thymic grafting into neonatally thymectomised (10, 19, 20) or lethally irradiated and thymectomised adult recipients (20). Such grafts have been shown, with the chromosome marker technique, eventually to be repopulated by host lymphocytes. Similar findings have been reported, using a serological technique for identification, when parental strain thymuses are grafted into F₁ hybrid hosts (9). Following grafting, cells of donor strain can be identified in the host' spleen (19, 20) and lymph nodes (10). Moreover, when grafted thymuses are regrafted into a second host strain, cells derived from the primary host again migrate to lymph nodes and spleen (11).

Irradiation experiments with partial body shielding, avoid the transfer of tissue from one animal to another. These have shown that if irradiation is carried out first with the lower half of the body shielded and then, one to three hours later, with the

upper half shielded the $L.D._{50}$ is doubled (3). Hence, during the interval between irradiations, cells capable of repopulating the thymus must have migrated from, presumably haemopoietic sites in the lower half of the body. If only the spleen is shielded instead of the whole lower half the same effect is produced (14, 15), presumably due to haemopoietic tissue in the organ.

Recently an extensive turnover of thymic lymphocytes has been demonstrated by means of parabiosis; one partner of a pair carrying a chromosome marker (12). Kinetic studies in normal animals, involving only the administration of tritiated thymidine, have yielded more circumstantial evidence in support of the theory of 'thymic cellular traffic'. The life span of thoracic duct, blood and lymph node small lymphocytes in a variety of species is of the order of 8 to 14 weeks (2, 4, 16, 24, 25) while that of bone marrow and thymic lymphocytes only 3 to 5 days (2, 24) suggesting that some relationship exists between the last named two cell types. However MITCHELL *et al.* (22) have commented on the remarkably high rate of proliferation of labelled thymocytes observed by them. Values for the rate of increase in the proportion of labelled cells in the thymus, reported both for rats (23) and for mice (21) following a single injection of tritiated thymidine also appear too rapid to be explained by cell division.

All the studies reviewed above have been carried out at the cellular level. We have approached the problem by measuring the changes in specific activity of the total DNA in the thymus following a single administration of tritiated thymidine. Since all injected thymidine is utilized within a period of approximately 30 min (18) any increase in specific activity of the DNA with time must result from a migration into the thymus of cells labelled elsewhere in the body. (The implications of the possible reutilisation of labelled DNA breakdown products are discussed later.) In the experiments described here we have specifically depressed thymic incorporation during the period of thymidine availibility in order to enhance any effect due to the immigration of labelled cells. We have used two methods to achieve this depression: irradiation and chilling.

Material and Method

Animals. 12 weeks old male mice of an inbred Swiss Albino strain, weighing 30 to 35 g were used; three to five mice for each experimental point.

Tritiated thymidine was obtained from the Radiochemical Centre, Amersham, and had a specific activity of 13.5 c/mm. Each mouse received 5.0 μc intraperitoneally. Except when otherwise stated, the mice were killed one hour after administration of thymidine.

Irradiation. 140 Kep X-rays, filtered through 0.1 mm Cu were delivered at a dose rate of 200 r/min, to a total dose of 500 r. The mice were anaesthetised with Nembutal during irradiation. When the thymic region alone was irradiated, the remainder of the body was shielded by a 3 mm thick sheet of lead.

Thymic chilling was achieved by means of a 'cold finger' (Fig. 1), which consisted of a thin-walled aluminium thimble, ½ in in diameter, attached by a short length of rubber tubing to a thickwalled glass tube, 4 in in length. Alcohol, cooled by passing through a coil of copper tubing immersed in an alcohol-dry ice bath, circulated through the 'cold finger' at a rate that could be controlled by a screw clamp. The 'cold finger'

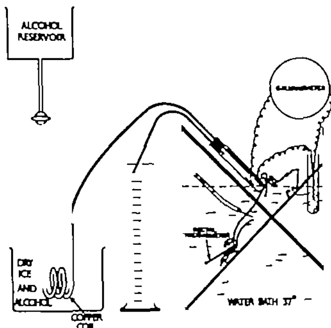


Fig. 1 Apparatus for cooling the thymus, showing the position of the 'cold finger' over the thymus and the arrangement for varying the rate of flow of cold alcohol and for recording the temperature of the chest wall. The animal is fixed with rubber bands (not shown) to the inclined perspex holder (shaded black) 3 cm in width. Details of the assembly are given in the text.

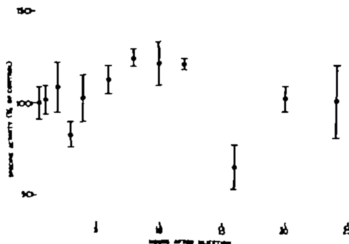


Fig. 2. Variations in the specific activity of DNA in the thymus over period of 24 h following single i/p injection of tritiated thymidine.

was placed in contact with the chest wall, over the thymus, and the skin temperature recorded by means of thermocouple. (Preliminary experiments with thermocouples introduced into the thymus itself had established that the thymic temperature could be adequately assessed by a thermocouple interposed between the 'cold finger' and the chest wall.) The required temperature was achieved by adjusting the rate of flow of alcohol. The body temperature was recorded by a rectal thermometer and was maintained by immersing the animal, anaesthetized with Nembutal, in water-bath at 37 so that the 'cold finger' was just above the surface. A sheet of in Perspex provided thermal insulation. Tritiated thymidine could be injected intraperitoneally through polythene capillary tubing attached to an in-dwelling needle.

Specific activity of DNA was determined by a method described previously (5). Tissue was homogenized in cold ethanol-saline and the precipitate successively extracted with ethanol, ether N perchloric acid at 0 and 0.5 N perchloric acid at 80 The DNA hydrolysed by hot perchloric acid was estimated spectrophotometrically by absorption at 260 mμ and the radioactivity determined in a Packard Tricarb liquid scintillation counter. Specific activities, after correction for body weight, were expressed as percentages of the mean values obtained for the control group of mice in each separate experiment.

Results

When tritiated thymidine was administered to normal mice there was no appreciable change in the specific activity of DNA in the thymus for the first 24 h. Therefore, either cells of the thymus that became labelled remained in that organ for more than 24 h before they—or their progeny—were discharged, or the loss of tritium labelled cells from the thymus was exactly balanced by the gain of labelled cells from other parts of the body The dip in the thymus curve of Fig. 2 at 16 h may reflect such a process.

Thymic irradiation. Two distinct aspects of the effects of irradiating the thymus were examined. One was concerned with the capacity of the thymus to continue to synthesize DNA following irradiation either of the whole body or of the thymus alone. The second was concerned with determining whether labelled DNA accumulates in the thymus after thymic DNA synthesis has been arrested by irradiation of the gland alone.

Fig 3 shows the effect of 500 r of X irradiation on the *total* DNA content of the thymus. Comparatively little change occurred during the first eight hours, but after 24 h there was a very profound drop. Moreover this sequence and the extent of the fall in DNA content was the same whether the whole body was irradiated or only the thymus.

The effect of irradiation on the capacity of the thymus to synthesize DNA is shown in Fig 4 After whole-body irradiation this capacity fell roughly exponentially to about 7 / of the normal

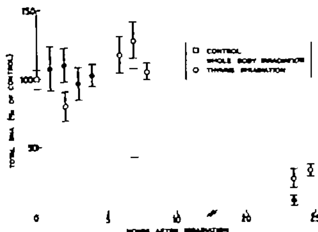


Fig 3. Changes in the total DNA content of the thymus following single dose of 500 X-irradiation, either to the whole animal (shaded circles) or to the thymus alone (open circles) Vertical bars represent one standard error

value at 7 h and was 4.5 / at 24 h. By contrast, when the thymus alone was irradiated the synthesizing capacity was only reduced to 50 / of the normal at 8 h and at 24 h it was still more than four times as high as after the whole body irradiation.

To examine the second aspect, tritiated thymidine was given three hours after irradiating the thymus alone, at which time the whole body irradiation experiment had shown that the capacity of the thymus to synthesize DNA had been reduced to about 30 %. The animals were killed 3 5 and 21 h later The specific activity of the DNA remained almost constant at about 30 / for the whole of this period (Fig 5) There was, therefore, no evidence for a net accumulation of labelled DNA in the thymus the profound fall in the *total* DNA of the thymus during this period (Fig 3) appears to have resulted equally from a loss of labelled as of un-labelled cells.

Thymic chilling The foregoing experiments were concerned with the functions of a thymus damaged by irradiation. To depress thymic activity temporarily and reversibly local chilling of the gland was attempted. *Fig 6 shows that the thymic temperature* recorded by a thermocouple buried within the gland drops rapidly on application of the cold finger and rises rapidly upon rewarming The capacity to synthesize DNA is also restored immediately (Table I) Fig 7 shows the effects of administering thymidine while the thymus was cooled and then of determining the specific activity of the DNA in the thymus at varying intervals thereafter

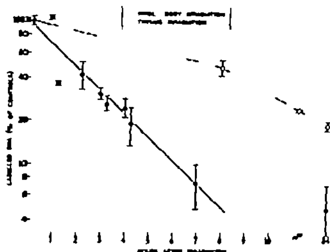


Fig 4 Rate of DNA synthesis in the thymus following 500 r X-irradiation, either to the whole animal (shaded circles) or to the thymus alone (open circles) Tritiated thymidine injected i/p at the times shown and animals killed one hour later Vertical bars represent one standard error

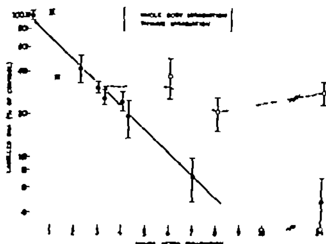


Fig 5 Variations in the specific activity of DNA in the thymus over period of 21 h following single i/p injection of tritiated thymidine given 3 h after 500 irradiation to the thymus (dotted line and open circles) The full line and shaded circles show how the capacity of the thymus to synthesized DNA falls after irradiation of the whole animal (Fig 4)

It will be seen that the specific activity was reduced to 23 6 / of the value in control animals during the period of cooling, but that the activity had risen to 63 / 4 h after re-warming, and to 126 /

Table

Effects of local cooling and of rewarming on the rate of DNA synthesis in the thymus

Thymic temperature °C	Thymidine administration	Rate of DNA synthesis % of control
23	During cooling	29.2
22	During cooling	35.4
22	During cooling	33.7
18	During cooling	17.5
12	During cooling	17.3
9	During cooling	16.8
4	Immediately on rewarming	187
4	5 h after rewarming	107

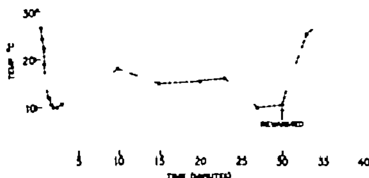


Fig. 6. Effect of applying cold finger' to the chest wall over the thymus on the thymic temperature. Temperatures were recorded by means of thermocouple buried within the gland.

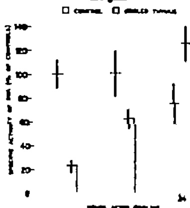


Fig. 7. Specific activity of DNA in the thymus during cooling, and 4 and 24 h after rewarming. A single i/p injection of tritiated thymidine was given while the gland was cooled.

24 h later In this study the control animals underwent the same experimental procedure, but cold alcohol was not circulated through the cold finger' Cooling the thymus had no effect on DNA synthesis in the spleen.

Discussion

Since the bulk of injected tritiated thymidine is utilised in the body in less than an hour any increase in the total radioactivity of an organ at longer intervals after injection must result from a transfer of labelled material from other parts of the body In our studies, such accumulation of labelled DNA was demonstrated in the period following the specific depression of synthesizing activity in the thymus by local chilling The extent of this accumulation precluded the possibility that reutilisation of labelled DNA break down products released at other sites could have made a significant contribution. Even if reutilisation occurred, the contribution by the hypothetical labelled products to the specific activity of the thymic DNA would have been relatively small since such labelled products would have been greatly diluted by corresponding unlabelled products released simultaneously It is therefore concluded that the increase in specific activity of the thymic DNA observed in this experiment resulted from an influx of cells that had been labelled elsewhere in the body at the time that the thymic activity was depressed.

No net accumulation of labelled DNA was observed following the depression of synthesizing activity in the thymus by local irradiation. However there was appreciable restoration of synthesizing capacity as compared with that following whole-body irradiation, suggesting that after local irradiation cells capable of synthesizing DNA (and presumably therefore, of multiplying) migrated into the thymus from other sites. However an alternative explanation could be that a radio-sensitive humoral feed back mechanism, destroyed by whole body irradiation, stimulates cell proliferation in a depleted thymus.

The finding that labelled DNA accumulates in the thymus after local chilling but not after local irradiation requires some explanation. Chilling does not produce an appreciable change in the total cellular population of the thymus therefore, if the mean specific activity of the DNA of immigrating cells is higher than that of emigrating cells there will be a net increase in total labelled

DNA in the gland. Irradiation, however produces involution of the gland this, in turn, would reduce the numbers of cells able to immigrate into the gland as well as curtail their sojourn. Although these restrictions apply equally to pre-labelled as to potentially synthesizing cells, restoration of synthesizing capacity in the gland is, by virtue of the rapid and profound depression of this function (see Fig 4) a much more sensitive test of cellular influx than is the net increase in specific activity of the total DNA.

An estimate may be made of the rate of cellular influx from the experimental data. In the chilled thymus the specific activity rose from 23 6% to 63% of the normal value in 4 h (Fig 7) while in the irradiated thymus the rise was from 5% to 50% in 8 h (Fig 4) Whether the subsequent release of labelled cells is sequential or random, both sets of data indicate an average period of sojourn within the thymus *for these cells* of roughly 14 h. The results further indicate that the cells that migrate into an irradiated thymus are capable of proliferating within it.

It follows from these conclusions that estimations of generation times for thymic lymphocytes, calculated from autoradiographic data, are invalid. If a substantial proportion of the thymocyte population is composed of cells in transit, estimates based on grain count analysis may reflect largely the generation times of precursor (bone marrow?) cells. Similarly estimates based on labelling indices (i. e., on changes in the proportion of cells labelled, with time) may reflect merely the turnover rate of migrant cells in the thymus. The similarity in generation times of thymic and of marrow lymphocytes reported by CAFFREY *et al.* (2) supports this contention.

Acknowledgements. This work was carried out as part of study of 'secondary disease' initiated by Prof. D. W SMITHERS, Director of the Radiotherapy Department. We are very grateful to Miss E. M. HILLYER for clerical assistance.

Summary

The specific activity of thymic DNA has been determined in mice given single intraperitoneal dose of tritiated thymidine following whole-body irradiation, thymic irradiation and thymic chilling In normal mice the specific activity of the DNA remained unchanged for 24 h following injection. After 500 whole-body irradiation the DNA synthesizing capacity of the thymus fell more steeply than after irradiation of the thymus alone. The specific activity of the DNA labelled while the thymus was chilled by means of 'cold finger' was reduced to 23 6% of the normal but rose progressively on rewarming. These results were explained on the basis of an influx into the thymus of cells originating elsewhere in the body

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Morphogénèse des micronoyaux supplémentaires (pseudo-corps de Jolly) dans les cellules érythropoiétiques irradiées

Recherches [illegible] en contraste de phase

E. G. Rondanelli, A. Trenta, E. Magliulo, V. Vannini et G. Gerna

Assez souvent on observe dans les tissus hémopoiétiques, dans certaines conditions pathologiques, de cellules érythropoiétiques pourvues d'un ou plusieurs micronoyaux supplémentaires, en plus des noyaux principaux, ou de très petits corps de chromatine dispersés dans le cytoplasme (1). Il s'agit de véritables micronoyaux satellites, dont un cas particulier est celui des érythroblastes avec «pseudo-corps de Jolly» qui, comme le spécifie le préfixe «pseudo» ne doivent pas être confondus avec les vrais «corps de Jolly» (3). Tandis que ces derniers (7) sont des restes nucléaires correspondant à des phénomènes de dégénérescence du noyau à la fin de la maturation érythroblastique, les micronoyaux satellites des érythroblastes, ou «pseudo-corps de Jolly» possèdent une chromatine aux mêmes caractères de celle des noyaux principaux et peuvent se rencontrer à l'intérieur de cellules qui souvent sont encore très éloignées de ce stade de maturation érythroblastique où normalement on observe les vrais corps de Jolly. La morphogénèse des micronoyaux satellites n'ayant pas encore été complètement éclaircie, nous rapportons nos observations en contraste de phase sur la formation de «pseudo-corps de Jolly» dans des cellules érythropoiétiques irradiées. Ces résultats nous paraissent de la plus grande importance, vu le fait que les «pseudo-corps de Jolly» (micronoyaux satellites érythroblastiques) se rencontrent le plus souvent dans des érythropathies comme l'anémie biermérienne (1) l'érythropathie benzénique et la maladie érythrémique.

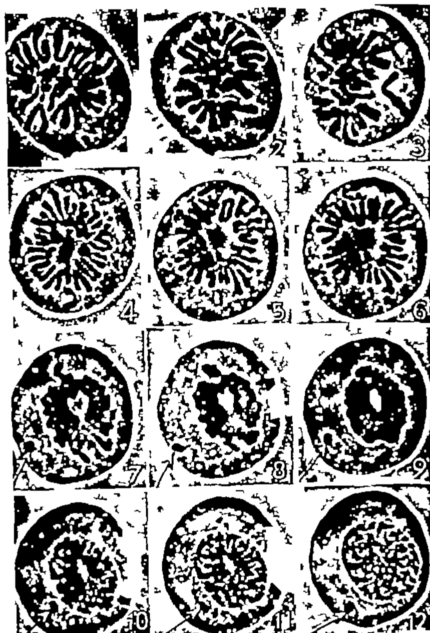


Fig. 1. Formation d'un micronoyau satellite à partir d'un chromosome (indiqué par la flèche) isolé au dehors d'une plaque métaphasique arrêtée par l'irradiation. 1–6. Plaque équatoriale avec quelques chromosomes aberrants. 7–9. Conglutination de la plaque équatoriale. Le chromosome aberrant indiqué par la flèche subit le même processus de homogénéisation de la chromatine. 10–12. Etapes de la reconstruction d'un noyau géant et d'un micronoyau supplémentaire.

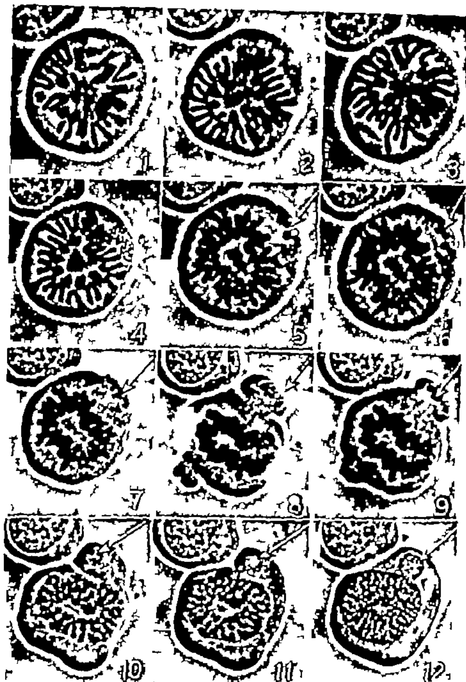


Fig. 2. Formation d'un micronoyau satellite à partir d'un groupement de chromosomes isolé au dehors d'une plaque métaphasique arrêtée par l'irradiation. 1–5. Plaque équatoriale. 6. Isolement d'un petit groupement de chromosomes. 7–9. Conglutination des chromosomes, pendant qu'une ébauche de sillon de division cytoplasmique ne parvient pas à séparer les deux groupements de chromosomes en deux cellules-filles. 10–12. Résorption du sillon cytoplasmique et reconstruction d'un noyau géant et d'un micronoyau supplémentaire.

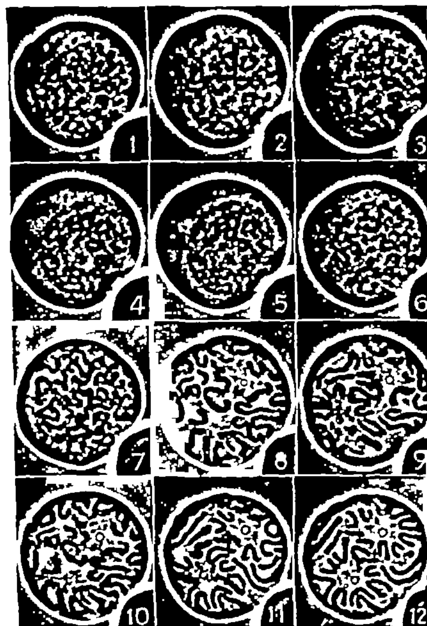


Fig. 3. Etapes de la formation d'une cellule géante à plusieurs micronoyaux supplémentaires (continue avec la fig. 4). 1–5 Prophase, 6–11 Métacinèse, avec bicentrosome métaphasique (pseudo-anaphase) à niveau des No. 9–11

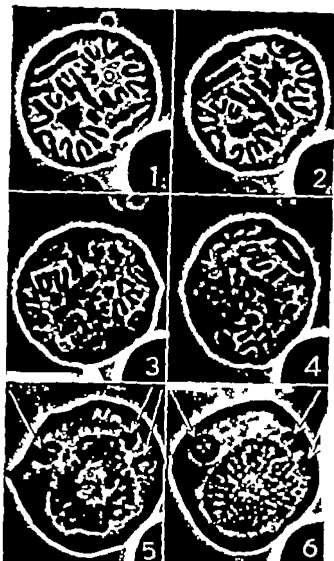


Fig. 4 Même mitose de la fig. 3. 1 et 2 Bicentrisme métaphasique avec chromosomes aberrants. 3 et 4 Dérangement complet de la figure métaphasique. 5 et 6 Reconstruction d'un noyau annulaire et de trois micronoyaux supplémentaires (indiqués par des flèches)

Matériel et Méthodes

On observé en contraste de phase et on effectué des prises cinématographiques de mitoses d'érythroblastes basophiles et polychromatophiles du *Triton Molge Vulgaris L.*, cultivés *in vitro* et irradiés avec 12 25 Pour l'obtention des cellules érythropoïétiques, la préparation des cultures, les données de l'irradiation et la technique de l'observation en contraste de phase et des prises cinématographiques, nous vous renvoyons au précédant travail de cytoradiobiologie (9)

Résultats

Nos observations montrent que les micronoyaux satellites et les «pseudo-corps de Jolly» peuvent se former par des troubles mitotiques qui conditionnent l'isolement dans le cytoplasme de chromosomes, ou petits groupements de chromosomes aberrants qui parallèlement à ce qui se vérifie dans le reste du matériel chromatique, mais à une échelle de dimensions mineures, s enveloppent d une membrane nucléaire, se décondensent en chromatine aux caractères interphasiques, et reconstruisent des véritables noyaux en miniature. On peut observer deux principales modalités morphogénétiques de micronoyaux supplémentaires

a) Une caryocinèse arrêtée en métaphase, soit avec isolement dans le cytoplasme au dehors de la plaque équatoriale d un (fig 1) ou plusieurs chromosomes réunis en petits groupements, avec leurs centromères rapprochés (fig 2) soit avec dispersion presque complète des chromosomes dans les cytoplasmes (fig 3 et 4) Ces chromosomes subissent ensuite des phénomènes de conglutination et de décondensation, semblables à ceux que l on observe normalement en ana télophase, mais se diversifiant de ceux-ci pour le fait qu ils se produisent dans des chromosomes aux caractères métaphasiques. Par conséquence des micronoyaux indépendants du ou des noyaux principaux vont se former à partir des chromosomes métaphasiques.

b) Une caryocinèse avec troubles de l'anaphase qui conditionnent soit un retard (ou un manque complet) de la migration d un chromosome ou de petits groupements de chromosomes, qui restent exclus du patrimoine génétique des noyaux se formant à partir des chromosomes ayant accompli normalement leur migration, soit des phénomènes de pluripolarité avec forte asymétrie de la migration chromosomique (fig 5 6 et 7) Ces chromosomes aberrants reconstruisent des micronoyaux satellites, indépendants du noyau principal (ou des noyaux principaux, si l anaphase était pluripolaire) La catachromasie chromosomique, différemment de ce qui arrivait dans la précédante modalité, se produit ici sur des chromosomes au caractères anaphasiques.

c) Une modalité intermédiaire aux deux précédantes, pour le fait que les chromosomes aberrants ne sont ni tous métaphasiques, ni tous anaphasiques, les micronoyaux pouvant être formés par un

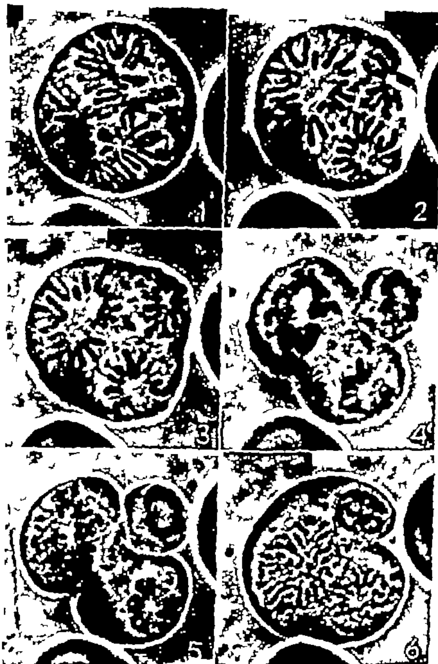


Fig. 5. Formation de micronoyaux par pluripolarité anaphasique avec asymétrie de la distribution des chromosomes. *1–3.* Anaphase tripolaire asymétrique. *4–6.* Formation de sillons cytoplasmiques qui néanmoins ne parviennent pas à séparer les trois cellules-filles. Les trois noyaux anisométriques, dont celui en haut est un vrai micronoyau, restent dans la même cellule.

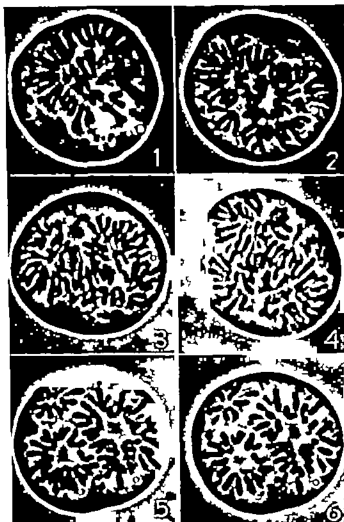


Fig. 6. Formation de micronoyaux par pluripolarité anaphasique avec asymétrie de la distribution des chromosomes (continue avec la fig. 7). On observe la migration asymétrique des chromosomes-fils dans trois étoiles.

mélange de chromosomes des deux types (fig. 8). Ces chromosomes étaient restés isolés probablement dans différents moments de la mitose atypique ou leur migration (dans le cas des chromosomes métaphasiques) avait été empêchée par des phénomènes inhibant la séparation des centromères-fils.

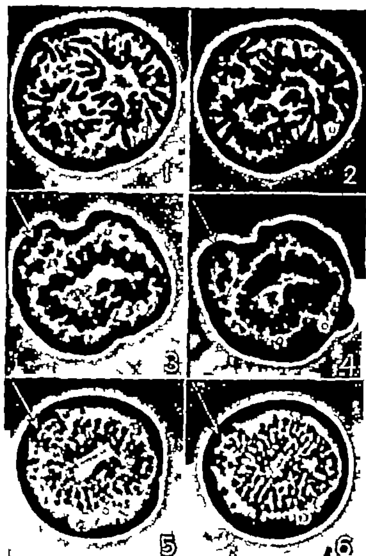


Fig. 7. Même mitose de la fig. 6. 1–4. Fusion de deux étoiles anaphasiques, conglutination réciproque des chromosomes. 5–6. Une ébauche de division cytoplasmique ne approfondit pas dans la cellule. Un noyau géant percé au centre par persistance d'un résidu des champs polaires centraux de l'étoile anaphasique, et un micronoyaux se sont formés.

Discussion

La formation de micronoyaux, ou de «caryomères» en différents types cellulaires avait été décrite par Tischler (11), Wilson (13) et Bessis (1). Ce phénomène avait été rapporté après action de

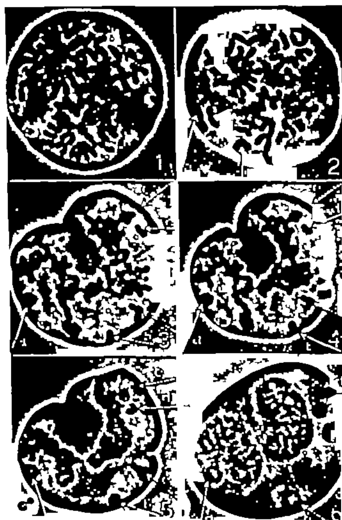


Fig. 8. Formation de plusieurs micronoyaux par chromosomes fils et chromosomes non divisés dispersés dans le cytoplasme ou réunis en groupements. Les flèches et dans la photo 2 indiquent deux couples de chromosomes-fils aberrants. 3–6 La conglutination qui tient sous les chromosomes réunis en groupements que les chromosomes dispersés porte à la formation de deux noyaux plus 5 micronoyaux, ces derniers et les chromosomes originaires étant indiqués par les mêmes flèches.

l'éther (13) des rayons X (8) et de la podophyllotoxine (2). SCHWARZ (10) avait décrit dans les cellules érythropoïétiques deux types de micronoyaux l'un par inhibition de l'activité du fuseau en méta

phase avec disposition des micronoyaux en couronne, et l'autre, par inhibition du fuseau avant la métaphase, avec la dispersion complète dans le cytoplasme des micronoyaux.

DISCOMBE (4) et BESSIS (1) avaient soutenu l'origine des micronoyaux érythroblastiques («pseudo-corps de Jolly») par troubles de la mitose. DI GUGLIELMO (3) a magistralement décrit les «noyaux secondaires» dans les érythroblastes de la maladie érythrémique dont il a mis en évidence les différences vis-à-vis des vrais corps de Jolly les premiers résultant, selon lui de *chromosomes aberrants et isolés*, contenus le plus souvent dans des cellules immatures et donc encore capable de mitose. FLIEDNER *et al* (5) ont soutenu les mêmes concepts, cependant sans les prouver pour l'origine des micronoyaux qu'ils avaient observé dans les cellules hémopoïétiques de sujets irradiés accidentalement. En outre ces micronoyaux étaient capables d incorporer l'H^3-thymidine ce qui prouve la capacité de leur ADN de se rédoubler Nos observations en contraste de phase montrent définitivement que les petits noyaux supplémentaires que l'on observe dans les cellules érythropoïétiques immatures, et qui ont été définis par les noms de «pseudo-corps de Jolly» «faux corps de Jolly» caryomères, «noyaux secondaires» etc. résultent de chromosomes (ou fragments de chromosomes) isolés au dehors des groupements principaux en métaphase ou en anaphase dont ils ne suivent plus la dynamique mitotique. Cela s accorde avec ce que DI GUGLIELMO (3) avait entrevu, bien que des phénomènes atypiques de fragmentation de noyaux immatures ne puissent pas être exclus, quoique nous ne les avons jamais observés dans notre matériel irradié.

Pour ce qui concerne la pathogénèse de la formation des micronoyaux satellites, il résulte de nos observations dans *les cellules érythropoïétiques irradiées que* ou un *procès de simple* fragmentation du noyau interphasique puisse être exclus, des mécanismes mitotiques bien définis doivent être envisagés. Le premier est un pluricentrisme métaphasique ou une pluripolarité anaphasique avec forte asymétrie de la distribution chromosomique, probablement par une lésion des procès de redoublement des centres polaires. Mais cela ne parait pas très acceptable si on considère que souvent le dérangement mitotique a *été* observé immédiatement après l'irradiation ce qui semble indiquer mieux une lésion des fibres soit continues que chromosomiques) du fuseau, ou de la jonction cen-

tromérique des chromosomes au fuseau même. La lésion du cynétochore peut-elle seule expliquer la séparation d un chromosome isolé d une plaque équatoriale d autre part apparemment normale tandis qu une destruction partielle des fibres du fuseau peut cependant signifier qu un certain degré de polarisation est conservé, et expliquer l'isolement de petits groupements de chromosomes avec leur centromères accôtés. Pour une destruction totale du fuseau, avec perte de la polarisation on a la dispersion complète des chromosomes dans le cytoplasme Dans ce cas l attraction mutuelle des cynétochores joue un rôle assez relevant en maintenant un certain nombre de chromosomes réunis en petits groupements formant des micronoyaux Le dernier mécanisme est enfin la fragmentation chromosomique, avec impossibilité des fragments détachés de participer à la formation des noyaux principaux. Cela expliquerait la formation de ces très petits corps chromatiniens isolés dans le cytoplasme, pour lesquels le terme de micronoyaux pour le vrai ne parait pas complètement appliquable, bien qu ils constituent les plus petits membres de la famille des «pseudo-corps de Jolly»

Il nous reste à discuter la destination des micronoyaux satellites dans les cellules irradiées. Deux possibilités peuvent être envisagées 1 Pour des lésions graves provoquées par l'irradiation, elles sont destinées à ne pas se reproduire, ou à donner lieu à des mitoses abortives ou à dégénerer Les micronoyaux satellites pourraient alors être soit expulsés, ou être lisés, ou simplement rester comme tels. 2. Ces cellules pourraient être encore capables de se diviser Dans ce cas le problème se pose si les chromosomes des micronoyaux participent à la mitose. Il faut aussi tenir compte du fait que les cellules contenant les micronoyaux sont généralement polyploïdes, ce qui entrainerait des troubles particuliers de la distribution chromosomique dans les cellules filles, et éventuellement la perpétuation de souches cellulaires dysploïdes.

La vitesse de disparaison des micronoyaux satellites dans les tissus irradiés à été employée par HORNSEY (6) pour calculer en admettant que les cellules avec micronoyaux satellites puissent entrer en mitose régulièrement, le temps de génération de ces tissus. Il résulte clairement de notre discussion sur la destination des micronoyaux supplémentaires que de telles déterminations chronologiques sont d une valeur très limitée Dans nos observations prolongées, nous n avons jamais observé les cellules avec micronoyaux

dagegen höher, wenn auch nicht annähernd so hoch wie zu Beginn der Kalt- und Warmexposition. Die Mittelwerte der Kalt- und Warmfrösche unterscheiden sich bei 1 % Irrtumswahrscheinlichkeit signifikant voneinander

$$t = 3{,}16 \quad t \frac{10}{P} = \frac{28}{1\%} = 2{,}76$$

Geschlechtsunterschiede waren auch hier nicht nachweisbar.

Das Differentialblutbild entsprach in etwa den Angaben über die heimischen Anuren (18). Auffällig war das Auftreten von stabkernigen Neutrophilen bei denjenigen Tieren, die einer niedrigen Wassertemperatur ausgesetzt waren. Es fanden sich zwar im Mittel nur 0,5 % Stabkernige, jedoch bedeutet dies schon eine gewisse Linksverschiebung, da bei den übrigen Tieren gar keine Stabkernigen gefunden wurden.

Diskussion

Die durchschnittliche Zahl der Leukozytenkernanhänge von *Xenopus laevis*, die bei einer mittleren Temperatur von 20° C gehalten wurden, entspricht mit 3,0±0,5 auf 200 neutrophile Leukozyten etwa dem Wert der heimischen Anuren (3,5±0,2) mit Ausnahme derjenigen Werte, die im Frühjahr gewonnen wurden. Eine Unterscheidung der Geschlechter nach der Zahl der Kernanhänge bei neutrophilen Granulozyten ist nach unseren Befunden nicht möglich, da eine gleich große Häufigkeit bei den Geschlechtern vorhanden ist.

Demgegenüber glaubt SCHIFFNER (19) auf Grund der von ihm gefundenen unterschiedlichen Häufigkeit von Kernanhängen bei *Rana esculenta* (im Mittel 18,75 bei Weibchen, 5,23 Kernanhänge bei Männchen auf 200 segmentierte Leukozyten) eine Geschlechtsdifferenzierung vornehmen zu können. Derartig hohe Werte, wie sie von SCHIFFNER für Weibchen angegeben werden, wurden von uns vereinzelt bei den heimischen Froscharten nur während des Frühjahrs, dann jedoch bei beiden Geschlechtern beobachtet.

Schon früher wies der eine von uns (3) darauf hin, daß nach WITSCHI (23, 24) und GALGANO (7) bei *Rana temporaria* und *Rana esculenta* die Geschlechtschromosomen etwa gleich groß und morphologisch wenig voneinander unterscheidbar sind.

BARR (8) vertritt die Hypothese, daß die Unterschiede hinsichtlich der Häufigkeit von Kernanhängen bei den Geschlechtern durch die unterschiedliche Größe der Geschlechtschromosomen bedingt sind. Ausnahmen – also etwa gleiche Häufigkeit – müssen

dementsprechend bei denjenigen Tieren auftreten, die durch die annähernd gleiche Größe der Geschlechtschromosomen ausgezeichnet sind. Dies ist nun tatsächlich für die heimischen Froscharten der Fall. Die Befunde von LÜERS UND LÜERS (12) an Mäusen Ratten und Meerschweinchen, die RENTSCH *et al.* (18) an Mäusen und Meerschweinchen bestätigten, sprechen ebenfalls für die von BARR vertretene These. Diese Tierarten weisen ebenfalls keine Größenunterschiede der Geschlechtschromosomen auf. Die genannten Autoren stellten bei ihnen etwa die gleiche Häufigkeit von Kernanhängen sowohl beim weiblichen als auch beim männlichen Geschlecht fest.

Bei *Xenopus laevis* liegen die Verhältnisse gegenüber den heimischen Anuren etwas anders. Einmal sind nach CHANG UND WITSCHI (5) bei dieser Froschart die Weibchen heterozygot. Sie besitzen die Konstellation ZW wogegen die Männchen homozygot (ZZ) sind. Die gleichen Verhältnisse sind beispielsweise auch am Huhn gefunden worden. Andererseits stellten WEILER UND OHNO (22) neben den heterozygoten Verhältnissen beim weiblichen Krallenfrosch erhebliche Größenunterschiede zwischen den Geschlechtschromosomen fest. Nach neuen Ansichten (15 21) soll sich bei den Säugetieren nur das eine heteropyknotische Chromosom (hier ein X-Chromosom) in den Kernanhängen manifestieren und das Geschlechtschromatin bilden. Wenn dies auch bei den niederen Tierarten der Fall sein sollte, müßte beim männlichen Krallenfrosch das eine Z-Chromosom und beim Weibchen ebenfalls das entsprechende Z-Chromosom in den Kernanhängen festgelegt sein, um die gleiche Zahl von Kernanhängen bei beiden Geschlechtern erklären zu können. Das würde gleichzeitig bedeuten, daß die Ausbildung der Kernanhänge nicht grundsätzlich an die weiblichen Geschlechtschromosomen gebunden sein könnte. Damit stimmen die Befunde von OHNO *et al.* (14) überein, die nachweisen konnten, daß sowohl beim Hahn als auch beim Huhn ein Z (oder X)-Chromosom vom Vatertier stammt. Geschlechtsdifferenzen an den Leukozytenkernen sind bisher an Hühnern ebenfalls nicht gefunden worden (20 6, 1)

Die zunehmenden Temperaturen im Frühjahr lassen bekannt lich bei poikilothermen Organismen auch den Stoffwechsel ansteigen. So lag es nahe, die erhöhten Kernanhangszahlen beim Frosch im Frühjahr damit in Zusammenhang zu bringen, umso mehr als nach BURMEISTER (3) in verschiedenen Jahren der Beginn

dagegen höher, wenn auch nicht annähernd so hoch wie zu Beginn der Kalt- und Warmexposition. Die Mittelwerte der Kalt- und Warmfrösche unterscheiden sich bei 1 % Irrtumswahrscheinlichkeit signifikant voneinander

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Zusammenfassung

Leukozytenkernanhänge konnten bei *Xenopus laevis* mit durchschnittlich 3,0±0,5 auf 200 Zellen nachgewiesen werden. Ein Unterschied zwischen den Geschlechtern war nicht zu ermitteln. Langfristige Temperatursenkung (Stoffwechselherabsetzung) verringerte die Anzahl der Kernanhänge.

Summary

Nuclear appendages were found in an average of 3.0±0.5 per 200 leukocytes in *Xenopus laevis*. No difference between the two sexes could be distinguished. Prolonged reduction of temperature diminished the number of these nuclear appendages.

Resumé

Des appendices nucléaires ont pu être trouvés en moyenne dans 3,0±0,5 sur 200 leucocytes. Il n'y a pas de différence entre les deux sexes. Un abaissement prolongé de la température (diminution du métabolisme) diminue le nombre des appendices nucléaires.

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Alexander S. Wiener with Section by *Moshe Shapiro* Advances in Blood Grouping. II. Grune and Stratton, New York 1965. 434 p. Price: $ 12.50.

With the preceding two volumes ('Rh-Hr Blood Types' 763 pages, 1954 and 'Advances in Blood Grouping' 549 pages, 1961), Dr WIENER has selected for republication a total of 189 of his over 500 published communications. The titles of the present and preceding volumes are, to this reviewer misleading because both fail to review advances in blood grouping or include, except for three reports by SHAPIRO any serious contributions of others. These volumes, however are of value to anyone who enjoys reading Dr. WIENER's contributions or wishes to refer to them at frequent intervals.

The latest volume contains, in addition to reprints, a 12 page 'Preface and Introduction' that attempts to convince the reader of the importance of WIENER's blood group terminology a one page 'Closing Remarks' repeating the same plea, and five pages of 1960–1965 publication references. There is also an 'Appendix' subtitled, 'LANDSTEINER and WIENER Discovery of the Rh Factor' Written after a testimonial dinner and scientific meeting in honor of Dr PHILIP LEVINE, this appendix claims the priority right of LANDSTEINER and WIENER for the discovery of Rh, but gives credit to LEVINE for the discovery of the pathogenesis of erythroblastosis and the role of Rh in this process, and for first describing hr' c, k and Tj LW the actual specificity of heterologous anti-rhesus agglutinins, was omitted, even though many persons consider this a most perceptive LEVINE observation.

Dr WIENER's observations of facts have always been sound. In contrast, his opinions, even of himself, are sometimes unacceptable. For example, Dr WIENER states (p. 426) that he always consults sources and that his publications, therefore, 'are singularly free of misquotations and myths' However *errare humanum est*. On p. 443 of this volume WIENER cites the first page of the 1941 report on erythroblastosis by LEVINE, BURNHAM, KATZIN and VOGEL as evidence of LEVINE's agreement to the priority for discovery of Rh by LANDSTEINER and WIENER. On the second page, however is the evidence that the 1939 LEVINE and STETSON case had anti-Rh in her serum. By not turning the page Dr WIENER felt free, in this appendix, to suggest that anti-A₁ rather than anti-Rh, might have been involved in the LEVINE-STETSON case. The truth of the matter is that this case was Rh-negative and had Rh antibodies. But the cases in 1936 of CULBERTSON and RATCLIFF cited in this appendix, were also Rh-negative when they were tested later by LEVINE (personal communication) while agglutination of human cells by rabbit anti-rhesus serums was reported in 1933 by LEON BUCHBINDER (J. Immunol. 25. 33).

OSLER said, 'In science the credit goes to the man who convinces the world, not to the man to whom the idea first occurs' Credit for human Rh (Rh_o, D or Rh1) pleomorphism belongs to LANDSTEINER and WIENER, who convinced the world with reliable agglutinating reagent, just as COOMBS did for antiglobulin reactions that had been discovered many years previously by MORESCHI. R. E. ROSENFIELD New York

Paul Kallós and B. H. Waksman: Progress in Allergy Fortschritte der Allergielehre, Vol. 9. S. Karger AG Basel/New York 1965, VIII + 308 S., 45 Abb., 9 Tab. Preis sF./DM 63.

Die erste Übersichtsarbeit von J. L. GOWANS und D. D. MCGREGOR über 'The

Dabei wird besonders eingehend das aktuelle Problem der Transplantationsreaktion erörtert. Unter kritischer Berücksichtigung der Literatur geben die Autoren eine klare Darstellung der doch recht komplizierten Materie, da sie eindeutig bewiesene Tatsachen von den noch mit Fragezeichen versehenen Erkenntnissen deutlich begrenzen.

Der zweite Beitrag von M. KAMINSKI über «The Analysis of the Antigenic Structure of Protein Molecules» ist deshalb wertvoll, weil besonders neuere Methoden, wie Gel-Diffusion und vor allem die von LAPRESLE eingeführte Methode des teilweisen enzymatischen Abbaues eingehende Berücksichtigung finden.

K. E. HELLSTRÖM und G. MÖLLER behandeln im 3. Artikel über «Immunological and Immunogenetic Aspects of Tumor Transplantation» experimentelle Untersuchungen, welche hauptsächlich an Mäusen durchgeführt wurden. Das sehr vielschichtige Thema der immunologischen und immunogenetischen Probleme der Tumortransplantation wird aus der Sicht des Spezialisten zur Diskussion gestellt. Wie die Autoren selbst betonen, kann es sich nicht um eine Gesamtübersicht über die heute bestehende Literatur handeln.

Im letzten Kapitel über «Immunosuppressive Drugs» von R. S. SCHWARTZ wird die Anwendung von Antimetaboliten und Alkylierungsmittel besprochen, welche in die normale immunologische Reaktion wie auch in die nichterwünschte Immunreaktion eingreifen können. Die Übersichtsarbeit enthält sehr viele Literaturangaben und ist durch die kritische Stellungnahme des Autors in bezug auf beobachtete experimentelle Wirkung und klinische Anwendungsmöglichkeit der betreffenden Medikamente gekennzeichnet.

Der vorliegende 9. Band der «Progress in Allergy» gibt einen Überblick über einige aktuelle Probleme der heutigen immunologischen Forschung und enthält eine Fülle von Literaturhinweisen, welche zur Einarbeitung in die verschiedenen Themen sehr nützlich sind.

Dr M. SCHWARZ-SPECK, Zürich

Varia

Normierung in der Hämatologie

Mitteilungen der Hämometerprüfstelle

Bekanntlich ist im Juli 1964 der Normenentwurf DIN 58931 Blatt 1 «Bestimmung des Gehaltes an Hämoglobin im Blut, Begriff, Maßeinheiten, Verfahren» erschienen. Es war im Rahmen des Deutschen Normenausschusses der erste Versuch, eine klinische Laboratoriumsmethode zu normen. Erfreulicherweise hat dieser Entwurf im In- und Ausland ein starkes und sehr positives Echo hervorgerufen, wobei in begrüßenswerter Weise auch kritische Verbesserungsvorschläge zu den einzelnen Punkten vorgebracht worden sind. Im März und Juni 1965 sind weitere Blätter dieser Norm als Entwurf erschienen.

Der Entwurf DIN 58931, Blatt 2, behandelt «Bestimmung des Hämoglobingehaltes im Blut, Anforderungen an die Reagenzien für die Hämoglobinbestimmung nach der Hämiglobincyanidmethode». Die einzelnen Punkte dieses Blattes führen den Titel 1 Zweck; 2. Begriff 3. Anforderungen 3.1 Reaktionskinetische Eigenschaften; 3.2 Gehalt an Kaliumferricyanid; 3.3 Toxizität 3.4 Haltbarkeit; 3.5 Konzentrate 3.6 Etikettierung und Verpackung 4. Literatur Anschließend folgen ausführliche Erläuterungen.

Der Entwurf DIN 58931 Blatt 3, behandelt «Bestimmung des Hämoglobingehaltes im Blut, Anforderungen an die Standardlösungen für die Hämoglobinbestimmung nach der Hämiglobincyanidmethode». Die einzelnen Punkte dieses Blattes sind betitelt 1 Zweck 2. Begriff 3. Anforderungen 3.1 Zusammensetzung; 3.2 Konzentration 3.3 Typische Farbkurven, 3.4 Bakteriologische Anforderungen 3.5 Haltbarkeit, 3.6 Abfüllen in Ampullen 3.7 Etikettierung und Verpackung; 4 Literatur Auch hier folgen Erläuterungen.

Der Entwurf DIN 58931, Blatt 4 behandelt: «Bestimmung des Hämoglobingehaltes im Blut, Prüfung der Reaktionslösungen und Standardlösungen für die Hämoglobinbestimmung nach der Hämiglobincyanidmethode». Die einzelnen Abschnitte haben den Titel 1. Zweck 2. Begriff; 2.1 Hersteller· 2.2 Prüfer; 3. Anforderungen an die Prüfer und an die Prüflaboratorien 4. Prüfverfahren für die Reaktionslösungen 4.1 Prüfung der Hämolysierbarkeit der Reaktionslösungen (diese und die folgenden Prüfungen sind jeweils aufgeteilt in «Prinzip» «benötigte Geräte» und «Durchführung») 4.2 Prüfung der Reaktionszeit 4.3 Prüfung der Umwandlung des Hämoglobins in Hämiglobincyanid 4.4 Prüfung der spektralen Eigenschaften der Reaktionslösung· 4.5 Prüfung des Ferricyanidgehaltes der Reaktionslösung 4.6 Prüfung des Zyanid-Ionen-Gehaltes der Reaktionslösung· 4.7 Prüfung des pH-Wertes der Reaktionslösung 4.8 Prüfung der Toxizität der Reaktionslösung; 4.9 Prüfung der Haltbarkeit, 4.10 Prüfung von Konzentraten 4.11 Prüfung der Verpackung und Etikettierung; 5. Prüfverfahren für Standardlösungen 5.1 *Prüfung der Standardlösung bei der Herstellung· 5.2 Prüfung* der spektralen Eigenschaften der Standardlösung; 5.3 Auswertung der Meßergebnisse 5.4 Prüfung der bakteriologischen Eigenschaften der Standardlösung· 5.5 Prüfung der Haltbarkeit; 5.6 Prüfung der Abfüllung in die Ampullen; 5.7 Prüfung der Etikettierung und Verpackung; 6. Literatur Wie bei den vorhergehenden, folgt auch hier eine ausführliche Erläuterung (mit durchgerechneten Beispielen)

Vor wenigen Monaten ist vom Deutschen Normenausschuß ein Arbeitsausschuß «Laboratoriumsdiagnostik» und im Rahmen dessen ein Unterausschuß Hämatologie gegründet worden. In diesem Unterausschuß sind Wissenschaftler (Mediziner Che-

niker Physiker) wie auch Delegierte der herstellenden Industrie vertreten. Der Unterausschuß hat zur Zeit 16 Mitglieder. Im kommenden Herbst wird dieser Unterausschuß an einer Sitzung die bis zu diesem Zeitpunkt eingetroffenen Einsprüche zu den Normenentwürfen DIN 58931 Blatt 1-4 eingehend besprechen und die endgültige Norm verabschieden.

In der nächsten Zeit beabsichtigt der Unterausschuß »Hämatologie« die Normung der Blutkörperchenzählung und der Hämatokrit-Bestimmung in Angriff zu nehmen. Die Normung weiterer wichtiger hämatologischer Methoden ist geplant. Anregungen, Vorschläge, Einwände zur Normungsarbeit in der Hämatologie sind zu richten an die Hämometerprüfstelle (78 Freiburg/Br., Hugstetterstraße 55) oder direkt an den Deutschen Normenausschuß (1 Berlin 15, Uhlandstraße 175).

K. G. v. Boroviczeny, Freiburg im Br.

Acta haemat. 35: 257–271 (1966)

Aus der 2. Medizinischen Klinik der Medizinischen Akademie (Prof. Dr. K. Oberdisse) und dem Zentrallaboratorium der Gesellschaft zur Bekämpfung der Krebskrankheiten (Prof. Dr. C. G. Schmidt), Düsseldorf

Die Aminosäurezusammensetzung normaler und pathologischer γG-Proteine

O. Wetter und Th. Hake

Die in quantitativer und qualitativer Hinsicht von den normalen menschlichen Serumproteinen fast immer deutlich abweichenden Paraproteine sind bald nach Einführung geeigneter vorwiegend elektrophoretischer Methoden und Fällungsreaktionen zu ihrer Isolierung auf die Aminosäurezusammensetzung untersucht und mit normalen γ-Globulinen verglichen worden (1, 2, 3, 4, 5, 6, 7). Die z.T. mit verschiedenen Methoden gewonnenen Ergebnisse stimmen im wesentlichen überein, werden aber unterschiedlich interpretiert. Einigkeit herrscht in der Feststellung des Fehlens ungewöhnlicher, d. h. in Serumproteinen normalerweise nicht vorkommender Aminosäuren in den Paraproteinen mit einer Ausnahme (5). Mit der Anwendung der Säulenchromatographie an Cellulose-Ionenaustauschermaterial (8, 9), der Immunoelektrophorese (10) und der Stärkegelelektrophorese (1) haben die Methoden zur Charakterisierung und Strukturaufklärung einzelner Serumproteine eine bedeutende Bereicherung erfahren.

Die immunoelektrophoretische Analyse paraproteinämischer Seren z.B. führte bald zu der Entdeckung eines 3. Immunglobulins, des γA-Proteins (12, 13, 14) und kürzlich eines 4. Immunglobulins, des γD-Proteins (15,16). Innerhalb der drei immunologisch unterscheidbaren Haupttypen von Immunglobulinen, der γG-, γA- und γM-Proteine konnten mittels der oben genannten Methoden strukturelle Besonderheiten festgestellt werden. Säulenchromatographisch (17, 18) und elektrophoretisch im Stärkegel (17) konnte die Heterogenität von γA-Paraproteinen gezeigt werden. Auch normales γG-Protein erwies sich unter geeigneten Bedingungen uneinheitlich. So konnte im Serum eines Falles von γA-Paraproteinämie bei der Fraktionierung an DEAE-Ionenaustauscher immunologisch reines γG-Protein in zwei Komponenten eluiert werden (18). Eine entsprechende Beobachtung wurde bei der Trennung von Kaninchen-γ-Globulin an dem gleichen Austauscher gemacht (19).

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In der Stärkegel-Elektrophorese zeigten auch γG-Paraproteine heterogene Zusammensetzung (17–20). Abgesehen von der individuellen Spezifität jedes γG-Proteins zeichneten sich die Paraproteine gegenüber den normalen γG-Proteinen durch die diskontinuierliche Verteilung des Materials über einen Teil des γ-Globulinbereiches aus. Dem entspricht immunoelektrophoretisch eine charakteristische Deformierung der γG-Präzipitate. Die Ursache dieses Phänomens der Diskontinuität der elektrophoretischen Verteilung von Paraproteinen (21) ist unbekannt. Versuche der Fraktionierung isolierter γG-Paraproteine an DEAE-Cellulose-Ionenaustauscher hatten bisher keinen Erfolg (22).

Kürzlich konnte gezeigt werden, daß elektrophoretisch isolierte γG-Proteine Gesunder und Plasmozytomkranker charakteristische Unterschiede in der optischen Drehfähigkeit aufweisen (23). Damit wurde zu den bekannten immunologischen und elektrophoretischen Eigenschaften in der Stärkegelelektrophorese ein weiteres Kriterium für die Paraproteinnatur von γG-Proteinen gefunden. Durch die nachstehend mitgeteilten Untersuchungen sollte geklärt werden, ob die gruppenspezifischen Eigenschaften normaler und pathologischer γG-Proteine einem charakteristischen physiko-chemischen Befund zugeordnet werden können und ob Unterschiede in der Aminosäurezusammensetzung zwischen beiden Gruppen bestehen.

Material und Methoden

Als Ausgangsmaterial für die Untersuchungen diente frisch gewonnenes Serum von 4 Gesunden und 8 Plasmozytomkranken sowie eines Patienten mit Makroglobulinämie Waldenström. Das Serum wurde entweder nach ein- bis zweitägiger Aufbewahrung bei 4 °C oder nach Einfrieren und Auftauen ausgiebig gegen Veronalpuffer pH 8,6, Ionenstärke 0,1 bei 4 °C dialysiert. Nach Verdünnung mit Veronalpuffer auf einen Proteingehalt von 2 bis 3 g% wurden die Proben in dem Spinco-H-Gerät bei 4 °C elektrophoretisch getrennt. Nach etwa 6stündiger Laufzeit bei 50 mA in der 60-ml-Zelle wurde das an dem hohen schmalen Gradienten kenntliche Paraprotein mit der Präparierkanüle aus dem absteigenden Schenkel der Tiselius-Zelle entnommen. Bei den Normalseren wurde als γG-Protein der vor dem Salzgradienten wandernde Gradient angesprochen.

Alle derartig isolierten Fraktionen wurden elektrophoretisch auf Membranfolien der Firma Schleicher und Schüll in dem Hochspannungsgerät der Firma Hormuth, Heidelberg, bei 1200 bis 1500 V und einer Laufzeit von 45 bis 60 min im Vergleich mit dem Vollserum untersucht.

Die Immunoelektrophorese wurde nach der von Scheidegger (24) angegebenen Methode in 2prozentigem Bacto- oder Noble-Agar der Firma Difco, Detroit, in Veronalpuffer pH 8,6, Ionenstärke 0,1 bei 40 V, 50 bis 60 mA und 60 bis 70 min Laufzeit durchgeführt. Als Antiseren wurden Kaninchenseren eigener Herstellung und solche der Behring-Werke, Marburg, benutzt.

Die Sedimentationsanalysen wurden in einer Spinco-Ultrazentrifuge Modell E bei 59780 RpM und 20 °C in der 12-mm-Zelle bei einem schlierenoptischen Winkel von 60° vorgenommen.

Für die Bestimmung der optischen Drehung diente das lichtelektrische Präzisionspolarimeter 0,005° der Firma Zeiß, Oberkochen. Die Messungen wurden bei Zimmertemperatur in der 2-cm-Zelle bei den Wellenlängen 365, 405, 436, 546 und 578 mμ vorgenommen. Lösungsmittel war in allen Fällen Veronalpuffer pH 8,6, Ionenstärke 0,1.

Die Auswertung der Meßergebnisse der optischen Drehfähigkeit wurde nach Lowry (25) und Moffitt (26, 27) in der an anderer Stelle (23) ausführlich dargestellten Weise vorgenommen.

Für die Eiweißbestimmung wurde die von Beisenherz (28) angegebene Modifikation der Biuretmethode benutzt.

Zur Bestimmung der quantitativen Aminosäurenzusammensetzung diente ein vollautomatisches Gerät der Firma Technikon. Methodisch wurde nach der von Spackman et al. (29) eingeführten Säulenchromatographie von Proteinhydrolysaten an Ionenaustauscherharzen in der Modifikation von Piez und Morris (30) vorgegangen. Als Eichlösung wurde ein Aminosäuregemisch der Firma Beckman Instr. benutzt. Die Proben wurden in 6 n Salzsäure 20 h bei 110°C hydrolysiert (31). Die Laufzeit der einzelnen Chromatogramme betrug 70 h. Die Werte für Cys/2 und Met. wurden aus ihren Sulfonsäure- bzw. Sulfoxydwerten zurückgerechnet, da keine genauen Vergleichswerte vorlagen.

Ergebnisse

In Tab. I und II sind die Ergebnisse der elektrophoretischen Untersuchungen, der Immunoelektrophorese, der Sedimentationsanalysen und der Bestimmungen der optischen Serumfraktionen zusammengefaßt. Die Proteinkonzentration betrug zwischen 1 und 8 mg/ml. Sowohl in der Trägerelektrophorese wie in der freien Elektrophorese handelte es sich in allen Fällen um einheitliches, in der γ-Region wanderndes Material. Wesentliche Unterschiede der Wanderungsgeschwindigkeit in den beiden Medien waren nicht festzustellen.

Immunoelektrophoretisch war in einer Probe (Co) eine Spur γG-Protein und in einer anderen Probe (We) eine Spur Transferrin als Beimengung zu dem Paraprotein nachzuweisen. In den Fällen Al und Mo handelt es sich um ein γA bzw. γM Paraprotein. Eine spezielle immunologische Analyse ergab, daß bei Al eine doppelte Proteinanomalie mit γG- und γA Paraproteinämie vorliegt.

Die Ergebnisse der Sedimentationsanalysen (Tab. I, II und Abb. 1, 2) zeigen, daß sämtliche γG-Paraproteine als einheitliche, schmalbasige Gradienten sedimentieren. Lediglich bei Bl setzt sich an der Basis des absteigenden Gradienten etwas schneller sedimentierendes Material ab (Abb. 2). Da immunoelektrophoretisch keine Beimengung anderer Serumproteine bestand, scheint es sich um eine Aggregation von 6,6 s-Molekülen zu handeln, wie sie noch deutlicher bei dem kommerziellen Präparat (Fr II der Firma Travenol, München) zum Vorschein kommt. Dieses immunologisch reine γG-Präparat enthielt außer der mit 6 s wandernden Hauptkomponente noch eine geringe Menge 8,5 s-Materials (Abb. 1). Zu einer derartigen Aggregation kann es im Verlauf der Präparation durch

Probe	D'phoretische Träger-E.	Bewegl. Freie E.	Immunoelektroph.	C in mg/ml	Sedimentationskoeffizienten bei 20°C	λc in mμ	[ ] 546 mμ	b_0	α-Helix %
Bo	γ	$γ_1$	γG-Protein	2,0	6,7 Spur schneller sedimentierenden Material	169	64,6	81,5	0
H	γ	γ	γG-Protein	1,5	5,7 Spur schneller sedimentierenden Material	246	49 7	—65,3	10,4
M	γ	γ + β	γG-Protein γM Spur Transferrin	3,1		182	64 4	87,8	0
Me	γ	$γ_1$	γG-Protein	0 1	6,5	214	68,9	53,1	0
Ra	γ	γ	γG-Protein	0 7	6,3	183	61,2	63,4	0
F	γ		γG-Protein	7 7	5,9 8,5	190	65,2	56,0	0

Tabelle II

Elektrophoretische Beweglichkeit, Immunoelektrophorese, Sedimentationskoeffizienten und optische Konstanten elektrophoretisch isolierter γG-Paraproteine.

Probe	D'phoretische Träger-E.	Bewegl. Freie E.	Immunoelektroph.	C in mg/ml	Sedimentationskoeffizienten bei 20°C	λc in mμ	[ ] 546 mμ	b_0	α-Helix %
Bi	γ	γ	γG-Paraprotein	3,0	6,2 Spur schneller sedimentierenden Material	205	57,2	6,9	0
Co	γ	γ	γG-Paraprotein	3,2	7,0	210	50,0	11,8	0
Ca	γ	γ	γG-Paraprotein	8,0	6,6	208	63,8	—15,4	2,4
He	γ	γ	γG-Paraprotein	3,0	5 1	212	81,8	23 4	0
Ko	γ	γ	γG-Paraprotein	6,8	7,5	209	60,8	18,0	0
Ti	$γ_1$	γ	γG-Paraprotein	2,0	6,8	217	79,0	—7 7	1,2
W	γ		γG-Paraprotein Transferrin	5 6	—	196	88,5	29,0	0
M	γ	γ	γA-Paraprotein	3,1	6,3 8,6 12,5	203	53,8	25,7	0
Mo	$γ_1$	$γ_1$	γM Paraprotein γG-Paraprotein	5,7	6,7 17 4 24,8	205	50,5	0	0

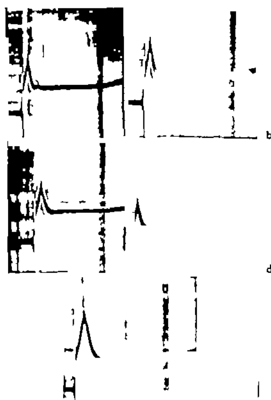


Abb. 1. Ultrazentrifugendiagramme normaler γG-Proteine. Sedimentationsrichtung von links nach rechts. Aufnahmezeiten in Minuten nach Erreichen der Höchstgeschwindigkeit 59 780 U/min, Schlierenoptischer Winkel 60°. Versuchstemperatur 20 °C. Konzentrationen s. Tab. I. Sonstige Versuchsbedingungen siehe Text. a) Bo. Aufnahmezeit 3 min, Sed. Koeff. 6,7 s. b) Ha. Aufnahmezeit 3 min, Sed. Koeff. 5,7 s. c) Me. Aufnahmezeit 5 min, Sed. Koeff. 6,5. d) Ra. Aufnahmezeit 3 min, Sed. Koeff. 6,3 s. e) F II. Aufnahmezeit 10 min, Sed. Koeff. 5,1 und 8,5

Fällung kommen. Entsprechende Beobachtungen sind bei bovinem γ-Globulin gemacht worden (32, 33). Auch die Proben Bo und Ha der Normalgruppe, immunologisch ebenfalls reines γG Protein, weisen Komponenten mit Sedimentationskoeffizienten größer als die 6,7 bzw. 5,7 s Hauptkomponente auf. Die genauen Werte der Sedimentationskoeffizienten ließen sich wegen zu geringer Konzentration nicht bestimmen.

Die Sedimentationskoeffizienten der normalen und pathologischen γG Proteine liegen im übrigen zwischen 5,1 s und 7,5 s. Während hinsichtlich der Sedimentationskoeffizienten kein Unter-

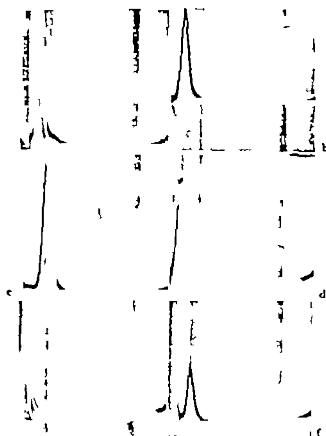


Abb. 2. Ultrazentrifugendiagramme pathologischer γG-Proteine. Versuchsbedingungen s. Abb. 1, Tab. II und Text. a) Bl. Aufnahmezeit 10 min, Sed. Koeff. 6,2 s. b) Co. Aufnahmezeit 13 min, Sed. Koeff. 7 s. c) Cz. Aufnahmezeit 21 min, Sed. Koeff. 6,6 s. d) He. Aufnahmezeit 8 min, Sed. Koeff. 5,1 s. e) Kü. Aufnahmezeit 6 min, Sed. Koeff. 7,5 s. f) Tl. Aufnahmezeit 7 min, Sed. Koeff. 6,8 s.

schied zwischen den beiden Gruppen festzustellen ist, besteht ein solcher augenfällig in der Form der Gradienten. Infolge größerer molekulargewichtsmäßiger Einheitlichkeit zeichnen sich die Paraproteine durch schmalbasige und hohe Gradienten im Gegensatz zu den breitbasigen, gedrungenen Gradienten der normalen γG-Moleküle aus. Die Proben Al und Mo sind sedimentationsanalytisch heterogen und weisen außer einer 6,3 bzw. 6,7 s Komponente schnellere Gradienten auf. Bei Al wurden die für γA-Paraproteine charakteristischen mittelschweren Komponenten (8,6 s, 12,5 s), bei Mo die für Makroglobuline typischen Sedimentationskoeffizienten (17,4 s, 24,8 s) gemessen.

Die Ergebnisse der Messungen der optischen Drehfähigkeit sind ebenfalls in Tab I und II aufgeführt. Die Auswertung der Drehwertbestimmungen nach LOWRY führt zu der Dispersionskonstanten λ_c Sie liegt für alle untersuchten Lösungen mit einer Ausnahme (Ha) in dem für globuläre Proteine des -Systems typischen Größenordnung von 170–217 mμ. Dieses Verhalten ist neben den schon genannten Kriterien ein weiteres Reinheitsmaß. Weiterhin sind die spezifischen Drehwerte bei 546 mμ angegeben.

Die Berechnung der optischen Konstante b_0 nach MOFFIT UND YANO ergab überwiegend geringe positive Werte oder 0 d.h. ein wesentlicher Gehalt an α-Helixstruktur war in dem untersuchten Material nicht vorhanden. Lediglich das durch seine hohe Dispersionskonstante von 246 mμ auffällige Präparat Ha hatte einen nennenswerten α-Helixgehalt (10,4) Durch Einsetzen von 246 anstelle von λ_0 in die Moffit-Gleichung ergab sich für b_0 ein positiver Wert (13,8) so daß in diesem Fall ein α-Helixgehalt durch sog Pseudokomplexibilität vorgetäuscht sein dürfte.

Die sich nach dem Verfahren von MOFFITT UND YANO weiterhin ergebende optische Konstante a_0 normaler und pathologischer γG-Proteine wird an anderer Stelle (23) mitgeteilt und sind deswegen hier nicht angeführt.

Die Ergebnisse der Aminosäureanalysen sind in Tab III für die Normalgruppe (I) für die γG-Paraproteine (II) für das γA und das γM Paraprotein (III bzw IV) zusammengefaßt. Zum Vergleich sind in Tab IV mit gleichartiger Methode gewonnene Ergebnisse anderer Autoren (35, 36, 37 38, 39 40) wiedergegeben. Da das untersuchte Material bei unserem Vorgehen in Veronalpuffer vorlag aus dem bei der Hydrolyse NH frei wurde, war eine Korrektur der Analysenergebnisse notwendig Ausgehend von der an 2 entsalzten Proben gefundenen Bruttozusammensetzung an Aminosäuren wurde der «Veronalfehler» eliminiert und für alle Analysen ein NH Wert von 3 zugrunde gelegt Er ergibt sich aus dem bei der Hydrolyse von Glu-NH_2 und Asp-NH_2 durch Zersetzung freiwerdenden NH_3.

Die Proben Me_1 und Me_2 stammen von verschiedenen Präparationen desselben Serums, bei Al_a und Al_b dagegen handelt es sich um eine Doppelbestimmung

Bei fehlenden Zahlenangaben war die Konzentration zu gering um auswertbare Ausschläge im Chromatogramm zu geben. Die Fehlerbreite der einzelnen Bestimmung beträgt etwa ± 5

Tabelle III

Aminosäurenzusammensetzung elektrophoretisch isolierter γG-Proteine Gesunder (I), γG-Paraproteine (II), eines γA-Paraproteins (III) und eines γM-Paraproteins (IV) in g% Aminosäurerest.

	I						II							III		IV
	Be	Ha	Mr_I	Mr_{II}	Ra	Tr_{II}	Bl	Co	Ca	He	Ka	T	W	Al_a	Al_b	Mo
Asp.	8.3	7.8	9.3	8.2	7.5	8.8	7.9	8.0	7.7	7.4	7.5	7.3	7.2	7.3	7.4	7.7
Thr	9.9	9.7	10.7	10.7	9.4	7.1	7.4	8.7	8.6	8.9	9.6	7.5	8.6	8.6	8.7	13.2
Ser	10.9	11.5	10.0	11.7	10.1	10.7	11.3	12.4	11.0	12.5	11.8	11.8	12.0	10.9	10.8	11.0
Glu.	10.1	9.9	10.3	8.2	11.0	11.8	10.5	11.0	11.0	8.3	11.5	10.3	9.6	11.0	12.7	10.0
Pro	7.0	7.4	6.0	6.9	7.1	10.0	7.9	7.3	7.6	10.2	6.7	9.6	8.6	8.7	6.5	6.4
Gly	6.8	6.9	7.2	7.3	7.0	4.9	6.5	6.7	6.4	7.1	7.0	8.1	7.0	7.0	4.7	6.6
Ala	5.4	5.2	5.9	6.2	5.2	4.1	5.7	4.8	5.7	4.3	5.3	5.3	5.5	6.8	5.4	6.0
Val.	7.2	7.3	6.5	7.8	8.4	5.6	7.8	7.0	6.6	6.9	7.1	8.5	7.3	6.9	6.8	6.6
Met	0.1	1.1	1.1	1.2	0.7	1.2	0.7	0.9	1.1	1.1	0.9		0.7	0.7	0.7	1.1
Cys/2	3.2	3.6	1.2		2.8	1.7	3.8	2.2	2.9	3.1	1.9		3.3	3.1	1.1	1.8
Ile	2.2	2.1	2.0	2.3	2.0	1.9	2.5	1.9	1.9	1.9	1.5	2.3	1.9	1.7	2.1	2.2
Leu	7.5	6.9	7.9	8.2	7.1	7.7	6.7	7.2	6.7	7.0	8.1	5.3	5.6	7.3	9.4	6.6
Tyr	3.4	3.4	3.3	3.5	3.4	5.1	4.0	3.8	3.9	3.8	5.5	4.2	3.9	2.8	1.0	2.9
Phe	3.4	3.4	3.7	3.5	3.1	3.9	4.0	3.4	3.3	3.2	3.6	3.3	3.1	3.1	3.1	3.4
NH	3.0	3.0	0	3.0	3.0	2.2	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Lys	5.7	5.9	5.4	6.2	5.8	6.7	5.1	6.1	6.0	5.8	5.2	7.6	6.3	4.8	5.8	4.8
His	1.9	2.0	2.0	2.1	1.9	2.4	1.5	1.8	1.8	1.5	1.3	2.1	1.5	1.6	4.0	1.0
Arg	3.1	2.9	1.9	2.9	3.0	3.1	3.1	3.3	3.6	3.6	3.3	3.5	3.8	3.8	3.7	3.1
Gluc NH	1.1		0.8		0.6	1.0	0.7	0.6	0.7	0.5	1.1					2.1

Tabelle IV

[illegible]ung von γG-Proteinen Gesunder (I) einem γG-Paraprotein (II) einem γA-Paraprotein (III) und einem γM Paraprotein (IV) nach Literaturangaben.

Aminosäure	I γG (33)	γG (38)	γG (37)	G (38)	G (39)	II γG (4)	III γA (40)	IV γM (8)	
Asp.	9.05	8.3	8.6	7.77	7.09	8.48	6.49	10.7	8.6
Thr.	8.90	8.2	6.3	7.01	7.18	6.41	8.19	8.7	9.25
Ser	11.75	12.2	10.4	9.15	9.09	10.16	8.25	8.9	8.4
Glu.	12.49	10.2	11.7	11.16	11.26	10.58	11.49	11.2	12.05
Pro	7.9	8.0	6.8	6.4	6.15	5.91	6.71	1.6	0.7
Gly	4.47	7.0	5.9	3.37	3.35	3.78	3.44	4.2	4.0
Ala.	4.05	5.4	3.8	3.29	3.18	3.88	3.86	5.1	4.15
Val.	9.42	9.0	8.5	7.92	8.1	8.41	6.61	10.2	7.7
Met.	0.90	0.5	1.1	0.95	0.95	0.97	0.91	1.3	1.05
Cys/2	2.26	2.2	2.2	2.07	2.2	1.83	2.66		4.5
Ile	2.59	2.1	2.3	2.16	2.14	2.3	1.76	3.3	3.3
Leu.	8.37	7.7	8.3	7.40	7.21	8.21	8.93	9.2	8.8
Ty	6.75	4.4	6.3	5.70	5.97	6.66	4.06	4.5	5.0
Phe	4.79	3.3	1.8	4.07	4.3	4.78	3.96	6.4	5.2
NH	1.75		4.02		1.46	1.26	Cy 0.31		
Lys.	8.01	6.5	7.4	7.00	6.96	7.42	4.81	7.1	5.6
His.	2.53	1.9	1.9	2.41	2.28	2.12	1.7	1.5	2.05
Arg.	4.45	3.0	4.3	4.02	1.02	4.91	4.76	7.0	6.4
Try	3.42			2.63	3.83	1.48			
Glu., NH_2					2.9	0.3	2.35		
Hex.						1.59	3.7		

Try wurde nicht bestimmt. Bei den Angaben für Cys/2 und Met ist mit einem Fehler bis 10 % zu rechnen weil diese Werte aus ihren Sulfonsäuren bzw Sulfoxyden zurückgerechnet wurden und für diese keine genauen Vergleichswerte vorlagen

Aus Tab III geht hervor daß weder für eine Aminosäure noch für Glucosamin charakteristische Unterschiede zwischen den untersuchten Gruppen bestehen Innerhalb der methodischen Fehlerbreite ist die Aminosäurezusammensetzung der normalen und *pathologischen γG Proteine gleichartig* Auch das γA Paraprotein Al weicht im Aminosäuremuster nicht von dem des γG-Proteins ab Bei dem untersuchten γM Paraprotein Mo ist ein relativ hoher Threoningehalt festzustellen.

In Tab IV sind mit gleicher Methode zur Aminosäurebestimmung gewonnene Ergebnisse anderer Autoren aufgeführt Die Isolierung dieser Fraktionen erfolgte größtenteils mittels kombinierter Fällungsmethoden In der Gruppe I bietet sich ein ähnlich einheitliches Bild wie in Gruppe I der Tab III d.h. normales γG-Protein zeigt unabhängig von der zu seiner Isolierung benutzten Methode gleiche Aminosäurezusammensetzung Ebenfalls keine wesentlichen Unterschiede ergaben sich für den Glukosamingehalt der normalen und pathologischen γG-Proteine. Glukosamin war in allen Proben nachweisbar konnte aber wegen zu geringer Konzentration in einigen Fällen nicht quantitativ bestimmt werden Das gleiche gilt für die fehlenden Methionin und Cysteinwerte bei Ti bzw Me.

Diskussion

Untersuchungen der Aminosäurezusammensetzung normaler und pathologischer γ-Globuline wurden zunächst an Serumfraktionen durchgeführt, die durch unterschiedliche elektrophoretische Beweglichkeit charakterisiert waren. Signifikante Unterschiede der Aminosäurezusammensetzung und der Endgruppen bei γ- und β-Plasmocytomproteinen wurden beschrieben (4) Die Hoffnung durch vergleichende Studien des Aminosäuregehaltes die Frage beantworten zu können, ob Paraproteine ihren Namen als normalerweise nicht vorkommende Serumproteine zu Recht tragen, erfüllten sich von einer Ausnahme abgesehen (5) nicht.

Nachdem durch immunologische Methoden mindestens 3 Komponenten des γ-Systems identifiziert werden konnten sind Aminosäureanalysen mitgeteilt worden (39) Während Angaben

über den Aminosäuregehalt normaler γG Proteine relativ häufig zu finden sind, wurden Untersuchungen an Paraproteinen offenbar seltener durchgeführt. Ausschlaggebend für unsere Untersuchungen war aber daß γG Paraproteine sich durch ihr optisches Drehvermögen von normalem γG unterscheiden (23) Es sollte geklärt werden, ob die Aminosäurezusammensetzung dieser Proteine Aufschluß über die Ursache der beobachteten optischen Eigenschaften gibt. Auch der Kohlenhydratgehalt war in diesem Zusammenhang zu berücksichtigen. Es zeigte sich, daß keine der am Aufbau des Globulins beteiligten Aminosäuren charakteristische gruppenspezifische Veränderungen aufweist Auch der Glukosamingehalt war in den Gruppen der normalen und pathologischen γG-Proteine nicht signifikant unterschiedlich. Offensichtlich hat auch die Tatsache, daß die Paraproteine im Gegensatz zu der Normalgruppe keine Antikörperwirksamkeit aufweisen, keinen entscheidenden Einfluß auf das Ergebnis der Aminosäureanalysen.

Durch einen von den normalen Verhältnissen auffällig und systematisch abweichenden Bruttogehalt an Aminosäuren sind demnach die zur Gruppenspezifität führenden Moleküleigenschaften der γG-Paraproteine nicht zu erklären Auch das ein charakteristisches immunelektrophoretisches und sedimentationsanalytisches Verhalten aufweisende γA und γM Paraprotein (Al, Mo) unterscheidet sich im Aminosäuremuster nicht wesentlich von den γG-Proteinen.

Durch Säulenchromatographie der Hydrolyseprodukte konnte bisher lediglich die Individualspezifität von Paraproteinen, die auch immunologisch nachzuweisen ist (41) bestätigt werden. Auch unterschiedlicher Kohlenhydratgehalt der Immunglobuline und der γG γA und γM Paraproteine wurde gefunden, ein Ergebnis, das schon früher mit chemischen Methoden erzielt worden war (42)

Versuche, durch physikalisch-chemische Untersuchungen und Fluoreszenzpolarimetrie Unterschiede zwischen spezifischen Kaninchen-Antikörpern gegen Kalbsthyroglobulin und normalem γ-Globulin vom Kaninchen festzustellen, hatten ein negatives Ergebnis (43 44)

Erst neuerdings beanspruchen säulenchromatographische Aminosäureanalysen in diesem Zusammenhang wieder erhöhtes Interesse. Es konnte gezeigt werden, daß Antikörper verschiedener Spezifität unterschiedliche Aminosäurezusammensetzung aufweisen.

Derartige Untersuchungen sind zunächst an Kaninchen-Antikörpern, die gegen synthetische Haptene gerichtet sind, vorgenommen worden (45, 46). Es ergaben sich signifikante Unterschiede im Gehalt an Arg, Ileu, Leu, Asp und Tyr. Die Zugehörigkeit der Antikörper zu einer der γ-Globulin-Allotypen prägte sich im Aminosäuremuster nicht aus (47). Für gereinigte spezifische menschliche Antikörper eines Individuums konnte kürzlich ein entsprechendes Verhalten für eine Reihe von Aminosäuren gezeigt werden (47). Unterschiede von 7 bis 32 Aminosäureresten zwischen Antikörpermolekülen verschiedener Spezifität wurden festgestellt.

Mit der auf BURNET zurückgehenden Anschauung von der Zellstammauswahl (clonal selection) und der Information über die Aminosäuresequenz bei der Antikörpersynthese in Anwesenheit des Antigens sind diese Befunde zunächst besser zu vereinbaren als mit anderen Theorien, nach denen die Antikörperspezifität durch Anpassung präformierter γ-Globulin-Polypeptidketten an das Antigen hervorgerufen wird (template theory). Den neuen Gesichtspunkten, die sich durch den Befund der unterschiedlichen Aminosäure-Zusammensetzung von Antikörpern verschiedener Spezifität ergeben, versucht eine modifizierte template theory gerecht zu werden (48). Danach ist die Antikörperspezifität auf eine direkte Veränderung des genetischen Codes durch das Antigen zurückzuführen.

Zusammenfassung

Die Aminosäurenzusammensetzung von 7 elektrophoretisch isolierten γG-Paraproteinen, einem γA- und einem γM-Paraprotein wird mit der von 5 normalen γG-Proteinen verglichen. Ein Unterschied konnte nicht festgestellt werden. Auch der Glucosamingehalt ist innerhalb der methodischen Fehlerbreite gleich. Die Ergebnisse stimmen mit den Angaben anderer Autoren über γG-Proteine, die durch Fällung isoliert wurden, gut überein. Die durch die Sedimentationsanalyse in der Ultrazentrifuge und durch die Immunoelektrophorese im Agargel nachweisbaren Unterschiede zwischen beiden Gruppen lassen sich mit dieser Methode der Aminosäurebestimmung nicht auf unterschiedlichen Aminosäuregehalt zurückführen. Es werden weiterhin die Ergebnisse der Messungen des optischen Drehvermögens der Proben mitgeteilt. Die Dispersionskonstante λ_c und die optische Konstante b_0 wurden ermittelt. Der aus b_0 errechnete α-Helixgehalt war in fast allen Fällen 0.

Summary

The amino acid composition of seven electrophoretically isolated γG-paraproteins, one γA- and one γM-paraprotein is compared with that of five normal γG-proteins. No distinction was observed. The glucosamine content is also identical within the margin of error of the method. This result agrees well with the findings of other workers on γG-proteins isolated by precipitation. The differences shown between these

two groups of proteins by ultracentrifuge sedimentation analysis and immuno-electrophoresis in agar gel cannot be attributed by this method of determination to differences in amino acid composition. The results of measurement of optical rotation potential are also reported. The dispersion constant λ_C and the optical constant b_0 were determined. The αhelix content calculated from b_0 was 0 in almost every case.

Résumé

La composition en acides aminés de 7 γG-paraprotéines isolées par électrophorèse, d'une γA-paraprotéine et d'une γM-paraprotéine est comparé à celle de 5 γG-protéines normales. Une différence ne put pas être constatée. Leur contenu en glucosamine est, dans le cadre de l'erreur-limite, semblable. Les résultats correspondent bien à ceux que d'autres auteurs ont obtenus par précipitation. Les différences constatées à l'aide de l analyse de la sédimentation par ultracentrifuge et à l'aide de l'immunoélectrophorèse sur gel d'agar ne peuvent pas être attribuées (selon cette méthode d'analyse des acides aminés) à un contenu variable d'acides aminés. Les résultats de la détermination du pouvoir rotatoire des protéines sont rapportés. La constante de dispersion λ_C et la constante optique b_0 ont été déterminées. Le contenu en α-hélix, estimé à l'aide de b_0, était presque dans tous les cas 0.

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Materials and Methods

Patients Investigated

Whole blood samples from 300 mothers without previous history of spontaneous abortion (the control group) were examined during pregnancy for the presence of cold-agglutinating antibodies to assess the normal incidence of this factor. From the same group 100 random samples were selected for the evaluation of serum complement. Similar investigations were also carried out on 222 pregnant mothers with a previous history of spontaneous abortion who also possessed the anti-Tj^a-like hemolysin.

In order that the full range of anti-Tj^a-like activity could be investigated, an additional six patients from the same abortion series, were selected at random for more detailed serological evaluation of the behaviour of the hemolysin in relation to other tests.

Routine antenatal tests for treponemal infection confirmed the absence of syphilitic conditions in the 522 mothers investigated during pregnancy.

Serological Procedures

( ) *The evaluation of cold-agglutinating antibodies.* This was determined by the ficin test using the slide method. T one drop of 1 percent concentration of commercial ficin in buffered saline was added one drop of serum which was previously inactivated at 56°C for 30 min. To this mixture was then added one drop of pooled group 0 red cells previously washed with normal saline (0.85% NaCl) to give final concentration of 10* of red cells. At the completion of one hour' incubation at 5°C the red cell agglutionates, if present, were examined at room temperature.

(*b*) *The evaluation of serum complement.* This test consisted of the preparation of optimally sensitised sheep red cells with commercial anti-sheep red cell hemolysin. T void possible variations in the value of serum complement due to prolonged storage it was necessary to test the serum samples within 24 h of collection.

Serial saline dilutions of serum to be examined were prepared in standard test tubes. T each of these dilutions was added an equal volume of 4% suspension of sensitised sheep red cells. After thorough mixing the contents of the tubes were placed for 1 h in 37°C water bath. The highest serum dilution showing definite hemolysis at the completion of this incubation period indicated the serum complement value of the blood sample under investigation.

(*c*) *The evaluation of the anti-Tj^a-like hemolysin.* The routine evaluation of the anti-Tj^a-like hemolysin, unless otherwise discussed in the results of this report, was carried out by the simple addition of 1 volume (0.5 ml) of red cells (2% saline suspension of group 0 Tj^a-positive red cells) to 1 volume (0.5 ml) of serum. In view of the observation that the anti-Tj^a-like hemolysin does not require sensitisation at 5°C prior to incubation, the test tubes were placed directly into 37°C water bath for one hour then centrifuged for two min at 1000 rpm. The degree of hemolysis was read by visual inspection of the supernate and recorded as 0 to 4, 0 indicating the absence of hemolysis and 4 complete hemolysis.

Results and Discussion

The cold agglutinating antibody properties of heat inactivated PCH serum have been extensively studied by many investigators as cited by DACIE (1) and probably the most important observation has been the consistent agreement concerning the presence of cold

agglutinating antibodies in addition to hemolysins. LEVINE *et al.*, (4) confirmed that the agglutinating type of cold antibody found in PCH serum as well as the hemolysin do not differ in their specificity for Tj^a-positive red cells. It is however not unusual to find PCH bloods possessing more than one antibody and the presence of anti I in addition to anti Tj^a should be considered possible in some instances, as pointed out by WEINER *et al.* (11)

With these observations in mind we investigated the frequency of cold agglutinating antibodies in normal pregnancies and compared these results with the occurrence of similar antibodies in threatened aborters producing anti Tj -like hemolysins. Table I

Table I
The incidence of cold-agglutinating antibodies among mothers possessing anti-Tj^a-like hemolysins and those lacking this hemolytic factor

Degree of cold-agglutinating antibody present in serum	300 control mothers lacking anti-Tja-like hemolysins without history of previous abortions		222 mothers with anti-Tja-like hemolysins, and history of previous abortions	
	No.	%	No.	%
No reaction	215	71.8	157	70.7
Weak to moderate reaction	50	16.6	40	18.0
Strong reaction	35	11.6	25	11.3
Total	300	100.0	222	100.0

shows the distribution of cold-agglutinating antibodies among 300 control mothers and 222 mothers with the anti-Tj like hemolysin. The identical incidence of mothers lacking cold-agglutinating antibodies, or showing moderate to strong reactivity in both series of patients, suggests that the presence or absence of cold-agglutinating antibodies is in no way associated with the occurrence of the anti-Tj^a like hemolysin.

The 25 samples of serum among the aborters (Table I) which strongly agglutinated random group O red cells at 5 C were also examined for their ability to sensitise pp (Tj negative) red cells. The positive results obtained in each case confirmed that the cold-agglutinating antibodies differed in specificity from the anti-Tj^a like hemolysin which was also present in the serum. This preliminary investigation would indicate that in the case of anti Tj^a like hemolysin production the simultaneous occurrence of cold-agglutinating antibodies does not appear to be a characteristic feature

The importance of serum complement in the serology of classical anti-Tj^a hemolysins has been well established. Its complete absence in the serum by either heat inactivation or the introduction of anti-complementary factors is known to prevent hemolysis from taking place. Among PCH patients repeated *in vivo* episodes of red cell hemolysis have also been reported to lower the serum complement concentration (2 9)

In the serology of anti Tj -like hemolysins the present study showed that the participation of serum complement does not appear to be a necessary mechanism for the activation of *in vitro* hemolysins. This observation was first established by examining blood samples in the presence of anticomplementary factors which was achieved by collecting the bloods into various anticoagulants. Table II shows the

Table II

The effect of various anticoagulants on the hemolytic potentiality of an anti-Tj^a-like and immune anti-A_1 serum.

Anti-coagulant used for the collection of blood samples	Hemolytic titer value of	
	Anti-Tja-like	Immune anti-A_1
Heparin	1 64	0
Mixed oxalates	1 64	0
Acid, citrate, dextrose	1 64	0
Sodium citrate	1 64	0
Controls whole blood	1 64	1 16

effects of this test for an anti-Tj^a like hemolysin as well as for a sample of immune anti-A_1 hemolysin. The findings showed that the degree of Tj^a-like hemolytic reactivity was in no way impaired by this procedure, whilst the immune anti A_1 hemolytic action was completely inactivated by the presence of anticomplementary substances.

The consistent ability of the anti-Tj^a like hemolysins to autohemolyse the patients' own red cells under *in vitro* conditions (10) suggested the possibility that the amount of complement present in *the serum may differ between aborters and nonaborters. Table III* records the frequency of serum complement values by our methods in a series of control pregnancy cases and mothers possessing the anti Tj^a like hemolysins. The apparently normal distribution of these values observed in the two groups of cases rejects the possible

Table III

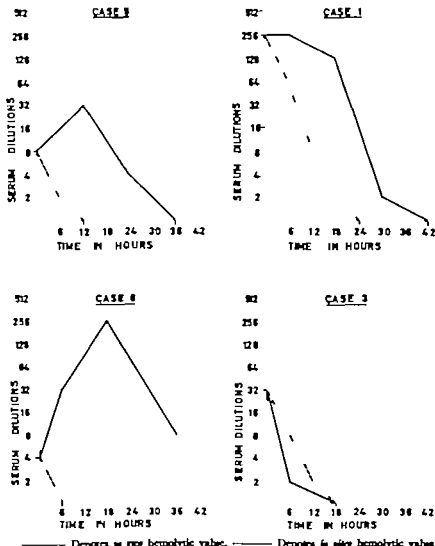
Serum complement value in patients with anti-Tj^a-like hemolysins and those lacking this factor

Serum complement activity expressed in dilutions	Control persons during pregnancy		Patients with anti-Tj^a-like hemolysins	
	No.	%	No.	%
1 : 2	—	—	—	—
1 : 4	2	2.0	4	4.1
1 : 8	25	25.0	32	33.3
1 : 16	49	49.0	44	45.8
1 : 32	14	14.0	10	10.4
1 : 64	10	10.0	6	6.4
1 : 128	—	—	—	—
All dilutions	100	100.0	96	100.0

association of serum complement as a likely mechanism for *in vivo* enhancement of hemolysis among aborters.

The effect of heat on the stability of PCH hemolysins has been reported to fluctuate in some instances by SIEBENS (8). However SCHUBOTHE (7) and HINZ *et al.* (3) conclude that most PCH hemolysins are probably not affected by heating at 56 C for 30 min. The results of similar studies on the behaviour of the anti Tj^a like hemolysin show that heating the serum at 56 C for 30 min completely destroyed the hemolytic activity. The subsequent addition of fresh serum complement of human or animal origin failed to restore the hemolytic action, thus indicating that the anti Tj^a like hemolysin is probably not thermostable.

Prolonged 37 C incubation of serum samples containing these hemolysins also resulted in complete loss of hemolytic activity. This observation suggested the possibility that the rate of *in vitro* elimination of anti Tj^a like observed at 37 C may in someway be associated with the characteristic ability of this hemolysin to appear disappear and reappear at frequent intervals under *in vivo* conditions (10). To evaluate this probability it was necessary to measure the degree of hemolytic activity at different intervals in a patient's serum incubated at 37 C against the degree of hemolytic activity recorded when freshly collected samples of blood were tested from the same person at a corresponding interval of time. Fig. 1 shows the results of several experiments of this nature. The assumption that the *in vivo* and *in vitro* rate of disappearance of the hemolysins could be controlled by constant exposure to the same temperature was not confirmed.



——— Denotes in vivo hemolytic value. ——— Denotes in vitro hemolytic value

Fig. 1 The behaviour pattern of anti-Tj^{a}-like hemolysins incubated at 37 °C (the in vitro hemolytic value) compared with the results obtained when fresh samples of blood were collected at corresponding intervall of time (the in vivo hemolytic value).

Under in vitro conditions the rate of hemolysin inactivation would appear to be directly related to the initial strength of the antibody whilst the in vivo behaviour shows typical curves of increased episodes of hemolysin production, followed in some instances by rapid

decrease in its intensity. The *in vivo* behaviour is in this sense referred to as the hemolytic value obtained immediately after the collection of blood. In case 3 the *in vivo* decrease in hemolytic activity appears even faster than the corresponding value recorded for the *in vitro* test. These observations are inclined to support the idea that the appearance of this hemolysin could be the result of active episodes of hemolysin production, induced by substances which perhaps originated from abnormal conceptions.

The ability of anti Tj^a like hemolysins to react directly against Tj positive red cells at 37°C without requiring the usual period of presensitization at the cold stage, not only differentiates them from the classical anti Tj found in Tj^a negative persons but also from the PCH patients possessing hemolysins of similar specificity. Although the optimum temperature for the direct hemolytic reaction of the anti Tj^a like hemolysin is 37 C, marked differences have frequently been noted. Table IV details the hemolytic action of six

Table IV

Degree of hemolytic reaction of anti-Tj^a-like in relation to temperature range and incubation time.

Case No.	37°C				22°C				5°C				Hemolytic titer value after 1 hour's incubation at 37°C
	min 15	min 30	min 45	min 60	min 15	min 30	min 45	min 60	min 15	min 30	min 45	min 60	
1	4	4	4	4	4	4	4	4	0	0	1	3	1 256
	4	4	4	4	1	3	4	4	0	0	0	2	1 128
3	0	3	4	4	0	0	0	3	0	0	0	0	1 32
4	0	0	3	4	0	0	0	2	0	0	0	0	1 16
5	0	0	1	3	0	0	0	0	0	0	0	0	1 8
6	0	0	0	3	0	0	0	0	0	0	0	0	1 4

4 = denotes complete hemolysis. 1 2 and 3 = partial degree of hemolysis. 0 = no hemolysis.

samples of anti Tj^a like hemolysins with titration values ranging from 1 256 down to 1 4. By testing the reactivity of these samples at various temperatures it was noted that the ability of this hemolysin to react against red cells at temperatures below 37 C appeared to be directly related to the initial strength of the hemolysin, the maximum degree of hemolysis usually being recorded at 37 C. Variations of these results could not be enhanced by prior immersion of the cell-serum suspensions at 5 C.

The positive direct and indirect antiglobulin reactions frequently obtained by testing samples of blood from PCH patients has been reported by DACIE *et al.* (2) to be due to the fixation of serum complement on the erythrocytes together with the hemolytic antibody. Thus complement attachment into red cells is demonstrated by the positive agglutination reaction of the red cells when examined against an anti-complement antiglobulin reagent.

The capacity of anti Tj -like hemolysins to destroy red cells *in vitro* without the interaction of complement as mentioned in the earlier part of this report, would present evidence to suggest that hemolysins which do not require serum complement will probably also fail to be sensitised by antiglobulin reagents. This was particularly noticeable when red cells sensitised by anti Tj -like hemolysins, which lacked the presence of cold-agglutinating antibodies, were examined against specific anti-alpha, alpha plus beta and gamma globulin serum samples. The consistent failure of the various antiglobulin reagents to react against red cells sensitised by anti-Tj^a-like hemolysins lacking cold-agglutinating antibodies could however also indicate that the antiglobulin reagents do not appear to be specific for the composition of the protein complex to which the anti Tj^a -like factor is associated.

By physicochemical and immunochemical studies, high titered cold-agglutinating antibodies are generally classified as beta 2M (19S) macroglobulins, which characteristically occur between the beta and gamma globulin region of the protein complex. WEINER *et al* (11) recently confirmed that the agglutinating factor of PCH antibodies is definitely associated with the 7S gammaglobulin complex. This would suggest that the hemolytic factor should also be found within the same protein fraction.

Investigations so far completed in connection with the location of the anti Tj^a-like hemolysin has confirmed Dr PHILIP Levine's original observation that the activity of this hemolysin is most intense between the alpha and beta globulin region (Fig 2). Repeated tests carried out on individual samples of serum containing the anti-Tj^a like hemolysin have consistently failed to show the presence of this hemolytic factor within the gamma globulin region of the fractionated protein.

Immuno-diffusion studies of the various electrophoretic fractions against specific anti-alpha, alpha plus beta and gamma globulin serum also confirmed that the fractions possessing anti-Tj^a

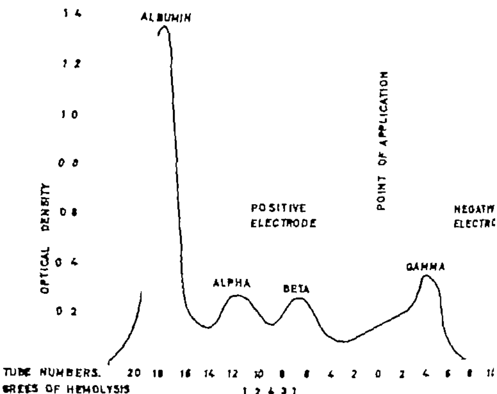


Fig. 2. Serum electrophoresis of an anti-Tj^a-like hemolysin for the evaluation of its position within the various protein bands.

like hemolysins were definitely only associated with the alpha and beta protein complex and not the gamma globulins.

Conclusions

Since the main object of the present study was to compare the serological behaviour pattern of the anti-Tj^a-like hemolysin with the konown pattern of PCH hemolysins, we have summarised in Table V the differences and similarities between the two hemolytic factors. The most significant deviations observed between the aborters in Western Australia and the PCH patients would appear to be the presence of cold-agglutinating antibodies, demand for serum complement, indirect antiglobulin reactivity thermolability and the established fact that PCH antibodies are associated with

Table V

Comparative reactivity of the hemolysin found in threatened aborters in Western Australia and PCH patients.

	Threatened aborters	PCH patients
1. *In vivo* autohemolytic reactivity	Always present	Always present
2. Specificity of hemolysin	Anti-Tj^a	Anti-Tj^a
3. Cold-agglutinating anti-Tj^a	Always absent	Always present
4. Demand for serum complement	Not associated with lytic activity	Always necessary for lytic activity
5. Indirect antiglobulin reactivity at 5°C temperature of sensitization	Always absent	Always positive
6. Thermolability of hemolysin	Always thermolabile	Always thermostable
7. Location of lytic activity in protein complex	Only present within the alpha and beta globulin region	Always present within the gamma globulin region

As quoted by DACIE (1) LEVINE *et al.* (4) and WEINER *et al.* (11).

gammaglobulins. From the point of view of specificity and *in vitro* autohemolytic reactivity these hemolysins do not appear to be different.

Since the topic concerning the serology of hemolysins is extremely large it would be impossible at this stage to review all the excellent work carried out in this field. But from the evidence presented in this report it is possible to conclude that the serological behaviour of the anti-Tj$_a$ like hemolysin appears to be significantly different from the classical varieties so far reported.

The observed variation could therefore facilitate the introduction of hypothesis which may or may not be significant with respect to the present interpretation of the mechanism of *in vivo* or *in vitro* hemolysis. It is for example possible to assume that the five dissimilar features recorded in this report between the anti Tj^a like and the PCH hemolysins may represent the basic differentiating pattern which divides the active form of autohemolysins observed among PCH patients from the "inactive" form of autohemolysins frequently found among the threatened aborters in Western Australia.

Although the absence of *in vivo* autohemolytic activity among the aborters has so far not been confirmed by current methods of

erythrocyte survival studies, it must be pointed out that the hundreds of abortion cases which have passed through this hospital have never seriously aroused clinicians suspicion that they were more anaemic than other normally pregnant women.

The observation that the anti Tj^a-like hemolysin appears to be located within the alpha and beta regions of the protein complex would indicate that this lytic factor can probably not be regarded as a classical antibody which as a rule resides either in the 7S or 19S gamma-globulin fractions. Until further evidence is available the decision whether the anti Tj -like hemolysin can be regarded as a true antibody must therefore remain in doubt.

MUSCHELL (6) has proposed that the anti Tj like hemolysin may possess properties of an acute phase reactant like the C-reactive protein which has a structure complementary to the C-polysaccharide of the pneumococcus and is not regarded as an antibody (5). *This hypothesis would regard the anti Tj^a like factor as part of a* structure which is complementary to classical Tj

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Summary

The serological qualities of anti-Tj^a-like hemolysins have been investigated to determine the extent of their common similarities or differences to the classical behaviour of paroxysmal cold hemoglobinuria hemolysins, which in previous accounts hav been reported to resemble in specificity and auto-hemolytic reactivity the hemolysins found in threatened aborters. Apart from the features already known to be alike this study also confirms that the hemolysins found in aborters do not require serum complement, are thermolabile, fail to be sensitised by anti-human-globulin serum, and lack the characteristic presence of cold-agglutinating antibodies of similar specificity as the hemolysins. *Further investigations also showed that these hemolysins are not located in the gamma-globulin region of the protein fraction. Their activity appears most intense between the alpha and beta regions only*

Zusammenfassung

Die serologischen Eigenschaften von anti-Tj^a-artigen Hämolysinen wurden mit denjenigen der klassischen Kältehämolysine der paroxysmalen Kältehämoglobinurie verglichen, die nach früheren Untersuchungen mit Bezug auf Spezifität und autohämolytischer Wirksamkeit den *Hämolysinen* bei *Frauen mit drohendem* Abort gleichen. Abgesehen von den bereits als ähnlich bekannten Eigenschaften bestätigte diese Untersuchung auch, daß die Hämolysine beim Abort kein Serumkomplement benötigen, thermolabil sind, mit anti-Human-Globulin-Serum nicht reagieren und das charakteristische Vorkommen von Kälteagglutininen gleicher Spezifität vermissen lassen. Weiter

bis ergab sich, daß diese Hämolysine nicht zur Gamma-Globulinfraktion gehören. Ihre stärkste Aktivität scheint zwischen der Alpha- und Betazone zu liegen.

Résumé

Les propriétés sérologiques d'hémolysines ressemblant au type anti-Tj^a ont été étudiées et comparées à celles des hémolysines à froid de l'hémoglobinurie paroxystique qui ressemblent selon des études antérieures quant à leur spécificité et leur activité auto-hémolytique aux hémolysines trouvées dans les cas d'avortement imminent. Cette étude a aussi confirmé à part les propriétés déjà connues pour étant semblables, que les hémolysines trouvées dans les cas d'avortement ne requièrent pas de complément sérique, qu'elles sont thermostables, ne réagissent pas avec de l'antisérum envers les globulines humaines et qu'il leur manque la présence caractéristique d'agglutinines à froid de même spécificité. D'autres recherches démontrèrent que des hémolysines ne font pas partie de la fraction des gamma-globulines. Leur activité la plus forte semble se situer entre la zone alpha et beta.

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Metabolic Aspects of Erythrocytes and Leukocytes in a Case of Congenital Non-Spherocytic Haemolytic Anaemia

E. STORTI, G. LEONCINI, A. TRALDI AND G. FORNAINI

During the course of the research that we have been conducting for many years (1 – 10) the case of a three-year-old boy is now added to our observations which clinical and hematological data have given a diagnosis of congenital non-spherocytic haemolytic anaemia.

We have examined some aspects of erythrocyte and leukocyte metabolism in this subject and in his family as we have already done in previous cases of a similar nature (4 6, 8) In particular the content and stability of GSH the utilization of glucose and the behaviour of the enzyme which conditions it or which are closely connected with it have been evaluated This research has pointed out pathological behaviour of the activity level and of the electrophoretic properties of G-6-PD of the subject under examination and of the female antecedents.

Case report

A. B. Propositus. At 3 months of age the patient colouring became pallid and he demonstrated asthenia, and splenomegaly At 5 months of age he was given blood transfusion which was initially well tolerated. Later the therapy had to be suspended due to violent febrile reactions and to rapid post transfusional haemolysis. At the time of admittance the pathological conditions were as follows RBC 1670000, Hb 52%, globular value 0.96, aniso-poikilocytosis, reticulocytes 15% erythroblastic ortochromatic 8–9%, mild-schistocytes, platelets 137000, WBC 7900, Serum bilirubine 1 63% with the Hymans van der Berg + direct ——— retarded + + indirect + + + Coombs test negative, non-pathological haemoglobin, non enhanced haemolysis before and after

The following abbreviations hav been used GSH reduced glutathione APH acetyl-phenyl-hydrazine; G-6-PD glucose-6-phosphate dehydrogenase 6-P-GD 6-phosphogluconate dehydrogenase GR glutathione reductase MR methaemoglobin reductase PK pyruvate kinase NADH reduced nicotinamide adenine dinucleotide; NADPH reduced nicotinamide adenine dinucleotide phosphate; NADPase NADP glycohydrolase.

The present work has been carried out under the Research Grant HE. 04586-06 of the National Institute of Health.

incubation. Bone marrow with erythroblastic hyperplasy and features of maturing inhibition. Nothing of note for the granuloblastic series.

Splenectomy has not modified the haemolytic process determining rather the appearance of the erythroblastosis. The patient' family (father mother paternal grandmother maternal grandmother maternal grandfather) do not demonstrate any haematologic diseases either from anamnesis or from up to date clinical examinations.

The examinations of the subjects used as controls were normal, for the referred experiments from pathological point of view or from hematic tests.

Research has always been carried out parallely on the pathological subject and on one control.

Materials and Methods

Blood was drawn with siliconated syringe, and then added to EDTA (2 mg/ml of blood) or heparin (3 U/ml) T separate the leukocytes from the erythrocytes, blood has been added in equal quantities to solution containing 3% dextran, 3% glucose, NaCl 0,9% and sedimented for an hour at 0 °C. The supernatant fraction constituted by the leukocytes was collected by suction then centrifuged for 10 min at 800 rpm. The supernatant was discarded and the cells suspended in 0.15 M KCl. The collected erythrocytes after sedimentation were washed 3 times with 0.15 M. KCl and suspended in the same solution. The lysis of the red and white cells was done by adding equal parts of water and freezing 3 times in mixture of dry ice/acetone. The removal of stroma was obtained by means of centrifugation for 30 min at 30 000 g. For the experiments of the utilization of glucose by erythrocytes, the incubation was performed in siliconated containers, whose internal dimensions were 18 × 36 mm (11) sealed by rubber stopper with vertical glass stick, at the end of which was fixed small piece of filter paper saturated with hydroxide of Hyamine to fix the $C^{14}O_2$. Incubation was performed at 37 °C in thermostatic bath, with shaking at 60 rpm. Before and at the end of incubation period, amounts of 2.0 ml of the reaction mixture were taken out, deproteinized with trichloroacetic acid (final concentration 10%) and centrifuged. The precipitate was then washed 3 times with 5% trichloroacetic acid, the supernatants were added again, neutralised with NaOH and brought to volume. Aliquots of these extracts were utilized for chemical determinations of glucose and lactic acid and for assays of the total radioactivity of lactic acid and of phosphorylated esters according to the method previously described (12).

For the experiments of the utilization of glucose in leukocytes the reaction mixture was placed in test tubes, and incubated according to methods described for erythrocytes. Before and after incubation 0.25 ml aliquots were taken out, deproteinized with trichloroacetic acid (final concentration 10%) and centrifuged The neutralised supernatant was brought to volume and utilized for chemical determination of glucose and of lactic acid.

The GSH content, before and after incubation with APH (13) has been determined by the technique of GROWDIT AND PHILIPS (14) glucose with glucose oxidase (15) lactic acid according to BARKER AND SUMMERSON (16).

The activities of G-6-PD and of 6-P-GD have been evaluated according to KORNBERG AND HORECKER (17) and HORECKER AND SMYRNIOTIS (18). The PK activity according to BÜCHER AND PFLEIDERER (19) GR and MR according to BOVENTVORE *et al.* (2) The NADPase has been prepared from rat spleen as previously described (20) one unit of NADPase is defined as that quantity of enzyme which splits 0.1 µmol of NADP in an hour at 37 °C.

The G-6-PD properties (sedimentation in sucrose gradient, electrophoresis on starch gel, thermostability affinity constants, pH Optimum, and inactivation) have been evaluated according to methods previously described (21).

The content in GSH is expressed in mg/100 ml of erythrocytes. For all enzymatic activities, excluding NADPase, one unit has been described as that quantity of enzyme which determines 1,0/min variation of optical density at 340 nm the erythrocyte specific activity is expressed in grams of hemoglobin, the leukocyte specific activity in grams of protein.

Spectrophotometric determinations were carried out with an Optica Spectrophotometer CF4 centrifugations have been done on Lourdes LRA and Spinco L2 Centrifuges; determinations of radioactivity on the Packard Tri-Carb Scintillator

Results

Table I refers to the behaviour of the GSH content and of the erythrocyte enzyme activities of the studied subject and his family We have taken for examination those enzymes which limit the oxidative shunt, i. e. G-6-PD and 6-P-GD which limits the balance between the oxidized and reduced forms of glutathione and of haemoglobin, i. e. GR and MR which in other cases of haemolytic anaemia showed pathological behaviour We have studied also PK activity alteration of which (22 23) characterises non-spherocytic haemolytic anaemia of type II of SELWIN AND DACIE (24) The only alteration observed has been that of G-6-PD of the propositus, of the mother and of the maternal grandmother the enzyme of these subjects has an activity level of 50% lower in comparison with controls. The utilisation of glucose by erythrocytes (Table II) was evaluated by the consumption of the monosaccharide, by the synthesis of phosphorylated esters and of lactic acid and by the liberation of CO_2 it did not present any variations in comparison with control subjects. The small number of reticulocytes present in the blood of the propositus excludes that the highest metabolic grade characteristic of the reticulocytes compensate a reduced activity of the old erythrocytes, both for the utilization process of glucose and for the enzymatic activity level, which are referred to in Table I. A recent research of ours (10) points out the presence of a direct relationship between the leukocytes and erythrocytes defect of G-6-PD in Italian subjects of different ethnical groups, sensitive to drugs and to fava beans. This has induced us to extend our investigations also to the leukocytes.

In all subjects examined the activity level of G-6-PD and 6-P GD and the utilization of glucose have been evaluated. It has been possible to follow this process only on the basis of the uptake of hexose and of the production of lactate, despite the small quantity of cells obtained The results referred to in Table III demonstrate

Table I
Glutathione content and enzymatic activities of erythrocytes.

…ts	GSH before	GSH after APH	G-6-PD	6-P-GD	TK	GR NADPH	GR NADH	…) NADPH
	72.3 ± 4.6	59.4 ± 5.7	15.3 ± 0.9	12.8 ± 0.8	13.8 ± 1.3	7 4 ± 0.6	1.6 ± 0.2	1 7 ± 0.3
…alon	70.7	53.2	7.2	13.3	12.2	6.6	1.9	2.0
	81.2	63.5	14.6	12.2	16.6	7.9	2.1	1.5
	75.3	64 1	5.4	12.5	16.3	7 1	1 4	1.7
…randmother	68.2	51 4	13.7	14 1	11.5	5.8	1.2	1.3
…grandfather	63.9	53.5	16.5	11.3	12.4	8.3	2.2	2.1
…grandmother	77 4	60.6	6.5	11.9	16.0	7.6	1.5	1.8

…I content is expressed as mg/100 ml of erythrocytes and the enzymatic activities as specific activi…
…s are reported in the text. The relative results to normal subjects are the means of 12 experi…
…se to pathological subjects of 3 experiments.

Table II
Glucose utilization of erythrocytes.

…ts	Metabolized glucose (a) µmol	Metabolized glucose (b) cpm (a + e … f)	(c) Phosphorylated esters cpm	Lactic acid (d) µmol	Lactic acid (e) cpm	(f) CO_2 cpm	% M… glucos… $\left(\frac{f \cdot …}{b}\right.$
	5.2	154 950	16 940	8.4	136 790	1220	[illegible]
…ostus	5.3	160 480	17 720	9.1	141 800	1160	[illegible]
	4.9	142 250	15 700	7.9	125 240	1310	[illegible]
	4 9	148 760	18 160	8.7	130 320	1280	[illegible]
…grandmother	5 5	165 010	17 380	9.2	146 500	1130	[illegible]
…l grandfather	4.7	148 960	14 930	8.1	132 800	1170	[illegible]
…l grandmother	5.9	158 850	17 930	8.5	139 630	1270	[illegible]

…e incubation mixture contained per ml 30 µmol of phosphate buffer pH 7.5; 10 µmol of glucose …
…th specific activity of 52.000 counts/minute/µmol 0.43 ml of erythrocytes. Incubation for 2 h at 3…
…ferred to ml of erythrocytes. For further indications see text. The relative results of normal subje…
…f 5 experiments, the relative ones to pathological subjects are the means of 2 experiments. Radio…
…ized glucose is lacking because it is not possible to determine the pyruvate radioactivity considerin…
…y 4–5% of transformed carbon as hexose is found again as pyruvate (enzymatic determinations) t…
…e. Although the experiments has been carried out in uniformly labelled substrate, $C^{14}O_2$ is good m…
… of metabolized hexose by oxidative shunt. The recyclization of pentatomic compounds by transket…
…olase is in fact very small in erythrocytes (12)

Table III

Enzymatic activities and glucose utilization of the leukocytes.

Subjects	G-6-PD	6-P-GD	Metabolized glucose	Synthesized lactate
Controls	156.4 ± 14	108.5 ± 12	246.8 ± 52.6	393.6 ± 82.4
AB, Propositus	140.3	111.4	302.8	437.8
Father	129.5	102.4	255.0	421.3
Mother	143.4	116.0	352.6	461.0
Paternal grandmother	122.2	100.6	321.2	384.8
Maternal grandfather	130.6	119.3	237.0	314.6
Maternal grandmother	157.5	114.2	286.6	496.8

Enzymatic activities are expressed as specific activity. For the glucose utilization experiments each ml of reaction mixture contained 30 µmol of phosphate buffer pH 7.5, 10 µmol of glucose · 10^7 leukocytes. Incubation for 2 h at 37 °C. The results are expressed in µmol 10^9 cells. For further indications see text. The results relative to normal subjects are the means of 8 experiments, the relative ones to pathological subjects of 3 experiments.

that the two enzymatic activities, and thus even the consumption of glucose and the synthesis of lactate do not present variations between subjects under examination and controls. Our studies show a single alteration that is a diminished activity level of the erythrocyte G-6-PD of the propositus and of the two female antecedents. In an attempt to clarify the nature of such modifications, the characteristics of the enzymatic protein of these subjects have been taken for examination. Beside the erythrocyte enzyme also the leukocyte enzyme has been taken for study because of the previously men-

Table IV

Properties of erythrocytes and leukocytes glucose-6-phosphate dehydrogenase.

Subjects	Cells	[illegible] at 37 °C per 60 min	[illegible] at NADPase (2 units)	Km G-6-PD	Km NADP	pH optimum	Electrophoretic mobility [illegible]
ols	erythrocytes	9	36	6.2×10^{-4}	2×10^{-4}	8.7	64
	leukocytes	34	62	3.1×10^{-4}	2×10^{-4}	9.1	37
ropositus	erythrocytes	10	37	6.4×10^{-4}	2.2×10^{-4}	8.6	65
	leukocytes	32	68	3.3×10^{-4}	1.8×10^{-4}	9.2	45
er	erythrocytes	8	40	6.0×10^{-4}	1.9×10^{-4}	8.7	62
	leukocytes	30	65	3.0×10^{-4}	2.0×10^{-5}	9.1	48
rnal grandmother	erythrocytes	8	35	6.3×10^{-4}	2.7×10^{-4}	8.8	68
	leukocytes	36	71	3.1×10^{-4}	2.3×10^{-4}	9.0	43

tioned relationship we have demonstrated between the two enzymes in the hemolytic anaemias with the defect of G-6-PD No modification has been revealed of the two enzymes (Table IV) relative to stability inactivation, affinity constants for substrate and co-enzyme, pH optimum, constant of sucrose sedimentation. Meanwhile for the erythrocyte enzyme, the electrophoretic migration in starch gel is also normal, the G-6-PD of leukocytes of all three subjects under examination reveal instead diminished migration

Discussion

On the basis of several cases of congenital non-spherocytic haemolytic anaemia which have been described by various authors (4 6 8, 22–37) it has been possible to conclude that these forms are amongst themselves heterogeneous for hereditary for clinical symptomatology and metabolic defects. A decisive contribution in classifying such forms was brought forward in 1952 by Selwyn and Dacie (24) by auto-haemolysis tests and the consequent subdivision into two types. Type I is characterised by normal auto-haemolysis or by slightly increased auto-haemolysis, but in any case conducted to normal by the addition of glucose or inosine It has not a serious symptomatology and generally with retarded appearance, and benefits the splenectomy It is not classifiable on the basis of a precise metabolic defect although many cases have a low level of a precise metabolic defect although many cases have a low level of G-6-PD activity Type II is instead characterised by greatly increased auto-hemolysis, by very precocious appearance, by seriousness of clinical symptomatology and does not benefit by the splenectomy Recent research has then demonstrated a diminished level of PK activity (22, 23) of the content in the phosphorylated esters and a diminished turnover of phosphate (29 22)

In the literature however numerous cases are reported which are unclassifiable into these two types because of a variety of clinical symptomatology and of metabolic defects and which can be classified as atypical forms (4 6 8, 30–33 37) The case which has been observed by us, can apparently be classified as Type II of Selwyn and Dacie, has instead demonstrated to be an atypical form. Despite the gravity of the clinical symptoms the characteristic features of the syndrome are in fact missing such as augmented auto-hemolysis, modifications of the PK activity and the content of

phosphorilated esters. In the propositus the only alteration observed is a diminished level of erythrocytes G-6-PD activity and a modification of electrophoretic behaviour of the same protein in the leukocytes. Similar modifications are observed in the mother and in the grandmother who never have presented a clinical symptomatology. However other properties of the erythrocyte enzyme are normal (pH optimum, constants of affinity and of sedimentation, ther molability and inactivation). Modified G-6-PD properties have been referred to by KIRKMAN (33) in cases of congenital non spherocytic haemolytic anaemia with intermedium level of enzyme.

Other metabolic aspects of the erythrocytes and the leukocytes of the studied subject and his family are normal. In the range of the physiological limits are the content of glutathione and its lability the activity of the systems regulating the balance between the oxidated and reduced forms of glutathione and haemoglobin, consumption of glucose, synthesis of phosphorylated esters, lactate and CO_2 production. In this subject, in the mother and in the grand mother the quantity of metabolised hexose by shunt is normal in spite of the diminished level of G-6-PD. Such behaviour can easily be explained because of the functional conditions of an enzymatic system are very different from whole cells and from ones which have been evaluated in reconstructed systems. In them are realised optimal conditions in concentrations of substrate, pH etc. In the case of G-6-PD when the activity is evaluated in the hemolysates, it has the capacity to oxidate 50 μmol of glucose per hour and per ml of erythrocytes. In the intact cell the rate of glucose metabolised per hour and per ml of erythrocytes by the oxidative shunt is 0.2 to 0.3 μmol. With this rate the erythrocytes needed in NADPH are assured. It is not therefore surprising that the diminished activity of G-6-PD as it is evaluated in the haemolysate has no meaning for the quantity of glucose metabolised by shunt.

In this sense our recent research has demonstrated (38) that in the erythrocytes of various ages which possess a G-6-PD activity notably different (10 times higher in young cells than in old ones) the quantity of oxidised glucose phosphate by shunt does not vary even when the total uptake of hexose is varied. In the present case as in those already described by us and in other cases referred to in literature, it is impossible to connect the cause of augmented hemolysis to an metabolic defect. The small number of the family does not allow an evaluation of the mechanism of transmission of the defect.

The present observations give only a further evidence of the heterogeneity of forms and of the difficulty of clarifying pathogenesis of the syndrome.

Summary

Some aspects of erythrocytes and leukocytes metabolisms have been studied in three year old boy affected by congenital non-spherocytic haemolytic anaemia, and in his family. The activities of glucose-6-phosphate-dehydrogenase, 6-phosphogluconate dehydrogenase, methaemoglobin-reductase, glutathione-reductase and pyruvate-kinase, glutathione content and stability and glucose uptake have been examined. The only metabolic alterations observed are those of G-6-PD of the propositus and his female antecedents. Erythrocyte enzymes have diminished activity and the leukocytes have modified electrophoretic properties.

Résumé

Le métabolisme des érythrocytes et des leucocytes a été étudié chez un garçon de 3 ans atteint d'une anémie hémolytique non-sphérocytaire et chez les membres de sa famille. L'activité de la glucose-6-phosphate-déshydrogénase de la methémoglobine réductase et de la pyruvat-kinase, la résorption du glucose, ainsi que le contenu en glutathion et sa stabilité ont été déterminés. Seule la glucose-6-phosphate-déshydrogénase du malade et de ses ancêtres féminins présenta des altérations métaboliques. L'activité des enzymes érythrocytaires était diminuée et les leucocytes avaient des propriétés électrophorétiques modifiées.

Zusammenfassung

Bei einem 3 Jahre alten Knaben mit kongenitaler nicht sphärocytärer hämolytischer Anämie und bei seinen Familienangehörigen wurde der Stoffwechsel von Erythrozyten und Leukozyten untersucht. Es wurden die Aktivitäten der Glukose-6-phosphatdehydrogenase, der 6-phosphoglukonatdehydrogenase, der Methämoglobinreduktase, der Glutathionreduktase und der Pyruvatkinase, sowie Gehalt und Stabilität von Glutathion und die Aufnahme von Glukose bestimmt. Die einzige Abweichung zeigte die Glukose-6-phosphatdehydrogenase des Patienten und seiner weiblichen Vorfahren. Die Aktivität der Erythrozyten-Enzyme war vermindert, und die Leukozyten wiesen ein abweichendes elektrophoretisches Verhalten auf.

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Separation of Lymphocytes from Human Blood

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The circulating lymphocyte has recently become a cell of particular interest, because of its possible role as an immunologically competent cell. A number of publications from the last three years seem to indicate that lymphocytes from human blood are able to react to the stimulation of added antigens *in vitro* with morphological transformation and possibly specific antibody production (2, 4, 5, 8, 9, 14).

The study of the *in vitro* reactivity of human lymphocytes has been hampered by the difficulties in obtaining pure lymphocyte cultures. Most investigations have been performed on mixed leukocyte cultures.

Previous methods of separating lymphocytes from granulocytes have been based on centrifuging cells in an albumin density gradient (17), a gelatin sedimentation technique (1), a magnetic technique using the phagocytic ability of granulocytes and monocytes for iron (13) or the adhesion of granulocytes and monocytes to glass wool (10) or to glass beads (7, 15). Most of the methods are rather complicated and there is always a risk that the cells are damaged by the procedure. A simple cotton wool filtration method for separating lymphocytes from heparinized blood was described by Fichtelius (6) and more recently used by Walker *et al.* (18).

The purpose of the present investigation was to test the efficiency and reliability of the cotton wool separation of lymphocytes from leukocyte suspensions, using a modification of Fichtelius' technique. Leucocytes were suspended in platelet poor fresh autologous plasma and the differential counts and yields of separated cells estimated. The standard technique was compared with modifications, using different media for the leukocyte suspensions and different types of cotton wool columns.



Fig. 1. Microphotograph of type 1 cotton wool fibers. Polarized light
Fig. 2. Microphotograph of type 2 cotton wool fibers. Polarized light

Material and Methods

Cotton wool columns were prepared from Pyrex glass cylinders, 100 mm in length and with an internal diameter of 13 mm. The cylinders were filled with weighed amount of bsorbent cotton wool. The standard columns contained 400 mg cotton wool

In a cylinder volume of 12 ml. Larger columns were prepared for the separation of lymphocytes from larger volumes of blood. The larger volumes contained 1400 mg absorbent cotton wool in cylinder volume of 45 ml (120 × 22 mm)

Two types of cotton wool were used in the experiments. Type 1 used in most of the experiments, showed *irregularly distorted cotton wool fibers (Fig. 1)* while type 2, used in one series of separations, had softer consistence with smooth and regular fibres (Fig 2) In another experiment small columns containing standard type cotton wool with varying tightness, weighing 200 mg, 400 mg and 600 mg, were used.

All glassware used was cleaned as for tissue culturing and sterilized by dry heat t 160 °C for 1 h. Rubber stoppers for the ends of the cotton wool columns were sterilized by autoclaving.

Heparinized venous blood was mixed with dextran and the erythrocytes allowed to sediment to obtain leukocytes in platelet rich plasma. Platelet poor leukocyte suspension was prepared by low speed centrifugation, as previously described (11) The leukocytes were gently resuspended in different media Ringer solution, stored pooled plasma, inactivated autologous plasma, fresh autologous plasma and fresh isologous plasma. Plasma was obtained from heparinized blood by high speed centrifugation and the volumes added to the leukocytes were equivalent to the volumes of leukocyte suspension before the separation of platelets.

The leukocyte suspensions were thoroughly mixed before being transferred to series of cotton wool columns. Six ml of leukocyte suspension were applied to the small columns, while the larger columns received 25 ml of cell suspension.

The columns were stoppered and incubated for 30 min t 37 °C. They were then washed through with olume of Ringer solution, equal to the volume of cells added to the columns. The washings were concentrated by centrifugation at 500 rpm (40 g) for 10 min. The cells were resuspended in AB serum.

Cell counts in the leukocyte suspensions and the concentrated effluents were estimated and smears made for differential counts. The methods of Dacie (3) were followed. The cell counts in the effluents were corrected for the changes in volume.

Results

Table I gives the yields of granulocytes and lymphocytes and the differential counts in the effluents from small and large columns that had received leukocytes suspended in fresh, autologous plasma The figures show that only about 1% of the granulocytes passed the small columns, while more than 50% of the lymphocytes were recovered. The proportions of lymphocytes found in the effluents varied within narrow limits. The total yield on the other hand varied from 60 to 100 % of the lymphocytes added to the columns. In the large columns the granulocytes were completely retained. The lymphocyte yields were lower than in the small columns. Due to the large size of the columns, however 8–10 × 10⁶ lymphocytes could be obtained from one separation

In Table II the results of separating lymphocytes from platelet rich versus platelet poor leukocyte suspensions are shown. All columns retained the granulocytes almost completely and the

Table I

Lymphocyte separation in cotton wool columns.

	Cell No. per mm³	Differential counts (%) granulocytes	lymphocytes	monocytes	damaged cells
Small columns					
Before separation	2800	70	25	1	4
After separation	750	3	86	0	11
	(500–950)	(2–4)	(84–90)		(7–14)
Large columns					
Before separation	2200	54	31	1	14
After separation	500	0	78	0	22
	(425–550)		(75–82)		(17–25)

	Yield all cells	granulocytes	lymphocytes
Small columns	26.8	1.0	91.4
	(18–34)	(0.7–1.8)	(60–108)
Large columns	22.7	0	56.6
	(19.5–25)		(46.7–60.5)

Mean and range from 5 columns.
Expressed as percentage of cells added to columns.

Table II

Lymphocyte separation from platelet-rich versus platelet-poor leukocyte suspension.

Suspension	Cell No. per mm³	Differential counts (%) granulocytes	lymphocytes	monocytes	damaged cells
Platelet-rich					
Before separation	1600	70	20	4	6
After separation	165	2	90	0	8
	(150–200)	(0–3)	(89–92)		(6–11)
Platelet-poor					
Before separation	1550	66	28	2	4
After separation	240	0	89	0	11
	(180–365)		(88–90)		(10–12)

Suspension	Yield** all cells	granulocytes	lymphocytes
*Platelet-rich**	10.3	0.3	46.5
	(9.4–12.5)	(0–0.5)	(42–55.5)
Platelet-poor	15.5	0	51.5
	(11.6–23.5)		(37.4–74.2)

Mean and range from 5 columns.
Expressed as percentage of cells added to columns

Table III

Lymphocyte separation from leukocytes suspended in Ringer solution, stored plasma and fresh plasma.

Suspension	Cell No. per mm³*	Differential counts ( ) granulocytes	lymphocytes	monocytes	damaged cells
Ringer solution					
Before separation	2200	72	16	4	8
After separation	710	48	31	0	21
	(670–750)	(46–51)	(30–32)		(17–24)
Stored plasma					
Before separation	2100	76	18	3	3
After separation	560	56	41	0	3
	(350–700)	(46–62)	(36–49)		(2 5)
Fresh plasma					
Before separation	2200	76	16	3	5
After separation	215	1	90	0	9
	(135–283)	(0–3)	(87–92)		(5–12)

Suspension	Yield** all cells	granulocytes	lymphocytes
*Ringer solution**	32.7	21 7	62.5
	(30.5–34)	(19.5–24)	(57–68)
*Stored plasma**	24	19	57.3
	(16.7 33.5)	(12 25.5)	(39–71)
*Fresh plasma**	9.8	0.15	54.4
	(6.1–13)	(0–0.7)	(34.5–74)

Mean and range from 2 columns.
Mean and range from 5 columns.
Expressed as percentage of cells added to columns.

effluents from all columns contained about 90 % lymphocytes, the rest being damaged cells. The yields of lymphocytes from the two leukocyte suspensions showed no significant difference

The effluents from the columns having received leukocytes in Ringer solution and in stored plasma showed a high percentage of granulocytes (Table III) The yields of lymphocytes showed no clear difference. There was no difference in the degree of granulocyte contamination or lymphocyte yield between the Ringer and stored plasma suspensions.

The separation of lymphocytes from leukocytes suspended in heat inactivated plasma gave results closely similar to those obtained from leukocytes in fresh plasma. No difference was found between cells suspended in autologous or isologous plasma (Table IV)

Table IV

Lymphocyte separation from leukocytes suspended in inactivated autologous plasma, fresh autologous plasma and fresh isologous plasma.

Suspension	Cell No. per mm³	Differential counts (%) granulocytes	lymphocytes	monocytes	damaged cells
Inactivated autologous plasma					
Before separation	1000	63	27	2	6
After separation	160	1	90	0	8
	(133–180)	(0–3)	(88–93)		(5–11)
Fresh, autologous plasma					
Before separation	1330	66	28	2	4
After separation	240	0	89	0	11
	(180–365)		(88–90)		(10–12)
Fresh, isologous plasma					
Before separation	1600	70	20	3	7
After separation	220	1	87	0	12
	(190–250)	(0–1)	(86–88)		(12 13)

Suspension	Yield* all cells	granulocytes	lymphocytes
*Inactivated, autologous plasma**	13.9	0.3	55
	(13.5–18)	(0–0.6)	(45.5–62)
*Fresh, autologous plasma**	15.5	0	51.5
	(11 6–23.5)		(37 4–74.2)
*Fresh, isologous plasma**	13.8	0.1	56.2
	(11 9–15.6)	(0–0.2)	(46.4–62.5)

Mean and range from 5 columns.
Mean and range from 2 columns.
Expressed as percentage of cells added to columns.

Lymphocyte separation in small columns containing cotton wool type 2 gave a high degree of granulocyte contamination (Table V)

Loose columns containing 200 mg of standard type cotton wool gave a significantly higher degree of granulocyte contamination than columns with 400 mg (standard columns) and 600 mg (Table VI) The lymphocyte yield was lower in the effluents from the tight columns.

Discussion

The main ad antage of the cotton wool separation method is its simplicity No complicated or expensive equipment is necessary The results also show that a complete separation of lymphocytes from granulocytes is possible. Although most erythrocytes are

Table V

Lymphocyte separation in small cotton wool columns, containing different types of cotton wool.

Cotton wool	Cell No. per mm³	Differential counts (%) granulo-cytes	lympho-cytes	mono-cytes	damaged cells
Type 1					
Before separation	2200	76	16	3	5
After separation	215	1	90	0	9
	(135–285)	(0–3)	(87–92)		(5–12)
Type					
Before separation	1500	80	16	2	2
After separation	370	26	61	1	13
	(300–435)	(21 29)	(58–63)	(0–1)	(11 17)

Cotton ool	all cells	yield granulocytes	lymphocytes
Type 1	9.8	0.15	54 4
	(6.1 13)	(0–0.7)	(34.5–74)
Type 2	24 7	8.0	91 7
	(20–29)	(6.9–10.5)	(73–109)

Mean and range from 3 columns.
Expressed as percentage of cells added to columns.

Table VI

Lymphocyte separation in small cotton wool columns of varying tightness.

	Cell No. per mm³	Differential counts ( ) granulo-cytes	lympho-cytes	mono-cytes	damaged cells
Before separation	2200	76	16	3	5
After separation					
*200 mg columns**	317	8	86	0	6
	(270–365)	(8–8)	(85–87)		(5–7)
400 mg columns	215	1	90	0	9
	(135–285)	(0–3)	(87 92)		(5–12)
600 mg columns	116	0	90	0	10
	(100–132)		(89–90)		(10–11)

	all cells	yield* granulocytes	lymphocytes
*200 mg columns**	14 4	1.5	77.5
	(12.3–16.6)	(1 3–1 7)	(66.7–88.1)
400 mg columns	9.8	0 15	54 4
	(6.1 13)	(0–0 7)	(34.5–74)
600 mg columns	5.3	0	23.6
	(4.5–6)		(25.6–35 6)

Mean and range from 2 columns.
Mean and range from 3 columns.
Expressed as percentage of cells added columns

separated by dextran sedimentation, some erythrocytes are always applied to the columns. Non-aggregated erythrocytes are not retained by the columns. The effluents therefore always contained a similar or higher number of erythrocytes than lymphocytes. Being non-nucleated cells with only slight tendency to morphological change and degeneration and relative low metabolic activity the erythrocyte contamination seems to be of less importance than contamination with granulocytes and thrombocytes.

The volume of cell suspension applied to the columns was of importance for the efficiency of lymphocyte separation. The small columns did not retain the granulocytes in more than 8–9 ml leukocyte suspension Thus was clearly dependent on the number of granulocytes and also somewhat dependent on the number of platelets in the leukocyte suspensions. It was also of importance not to increase the volume of the washing fluid over the volume of the cell suspension applied to the columns. A higher lymphocyte yield was obtained by increasing the washout, but the main purpose of the separation procedure was to obtain pure lymphocyte suspensions, the total number of lymphocytes obtained being of secondary importance.

The yield of lymphocytes was comparable to the values obtained by RABINOWITZ (15) with glass bead columns. The yields showed more variations than those obtained from glass bead columns. This is probably explained by the difficulties in obtaining standardized cotton wool columns. The volume and weight of the columns were constant, but there may probably have been variations in the tightness of cotton wool within each column with consequent variation in the retaining ability

Fresh plasma is essential for the adhesion of granulocytes to the cotton wool and therefore also for the separation of lymphocytes from granulocytes. Leukocytes suspended in stored plasma *gave no better results than cells suspended in Ringer solution. The* higher viscosity of plasma suspensions is therefore of minor importance. In the glass bead columns fresh plasma was also found to be essential for the retaining of granulocytes, but in contrast to our findings inactivated serum gave poor separation (15) This discrepancy may be due to a different mechanism for the adhesion of granulocytes to cotton wool fibers as compared to glass beads.

A fresh serum adherence factor has been found to enhance the ability of granulocytes to stick to a glass surface (15 16) This factor

appeared to be destroyed by heat-inactivation. Factors causing granulocyte adhesion to cotton wool fibers during incubation, how ever appear to be heat stable but storage labile.

The experiments with columns of different cotton wool types showed that there is a significant variation in granulocyte retaining ability. The cotton wool of type 2 microscopically showed a smooth and unbroken surface of the fibers compared to type 1 where the fibers were distorted and twisted. The latter type appeared to have a stronger attraction for the granulocytes.

The percentage of cells with damaged nuclear or cytoplasmatic membranes was quite high in the smears made from deposits after centrifugation of leukocyte suspensions and effluents. Phase contrast microscopy and acridine orange vital staining of the effluents showed a low degree of cell damage (12). Most of the smudge cells found in the smears therefore probably were caused by the spreading of the cells. Cell vitality may on the other hand, be reduced without obvious morphological change. Cell culture studies on lymphocytes separated in cotton wool columns with evaluation of cell vitality are to be reported.

Summary

A simple modification of the cotton wool lymphocyte separation method of Fichtelius is described. The separation gave almost pure lymphocyte effluents but the actual yield of lymphocytes was quite varyable. Fresh plasma was essential for the adhesion of granulocytes to the cotton wool columns. The efficiency of the separation was dependent on the type of cotton wool used and the tightness of the columns.

Zusammenfassung

Es wird eine einfache Modifikation der Baumwollmethode von Fichtelius zur Isolierung von Lymphocyten beschrieben. Die Trennung ergab einen nahezu reinen lymphocytären Abfluß, aber der wirkliche Ertrag an Lymphocyten war sehr wechselnd. Wesentlich für die Haftung der Granulocyten in der Baumwollsäule war Frischplasma. Die Ausbeute der Trennung war abhängig von der Art der verwendeten Baumwolle und von der Dichte der Säulen.

Résumé

Une modification simple de la méthode de Fichtelius servant à la séparation des lymphocytes, est décrite. La séparation donne un flux presque pure de lymphocytes, mais le rendement quantitatif en lymphocytes est très variable. L'emploi de plasma frais est essentiel pour l'adhésion des granulocytes aux colonnes de coton. L'efficacité du procédé dépend de l'espèce de coton employé et de la densité des colonnes.

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A New Technique for the Histochemical Demonstration of Phosphorylase in Blood Films

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Considerable interest is attached to the phosphorylase activity of leucocytes, since this enzyme in association with various de branching factors is responsible for the degradation of stored gly cogen. The enzyme catalyzes the following process by splitting 1 4 linkages between the glucose units of the polysaccharide molecule

$$\text{Phosphate} + (\text{glucose})_n \rightleftarrows (\text{glucose})_{n-1} + \text{glucose-1 phosphate}$$

Under *in vitro* conditions this reaction is readily reversed with the result that polysaccharide in the form of unbranched chains of glucose units is formed from glucose-1 phosphate, but *in vivo* gly cogen synthesis possibly only takes place via a different pathway involving uridine diphosphate glucose (10)

A reliable technique for the histochemical demonstration of phosphorylase in tissue sections was first developed by TAKEUCHI AND KURIAKI (7) The sections were incubated in a medium con taining glucose-1 phosphate, and an iodophilic polysaccharide was synthesised in cells possessing phosphorylase activity Addition of muscle adenylic acid and a small amount of glycogen as a primer was found to stimulate the reaction.

The histochemical demonstration of phosphorylase activity in blood films has, however presented difficulties, since direct appli cation of TAKEUCHI's medium on air-dried films causes cell damage which prevents the reaction (9) To overcome this difficulty brief prefixation of the blood films either in methanol (6, 8) or in dilute acetone (3) has been advocated In our hands, however these methods give inconstant staining results due to enzyme destruction during fixation

We therefore developed the following technique for the haematological application of the phosphorylase reaction, which permits the use of unfixed air-dried blood films, due to the protective effect of a high concentration of polyvinyl pyrrolidone in the incubating medium.

Method

Incubating Medium

The incubating medium, which was based on ERÄNKÖ and PALKAMA's medium (2), was composed as follows

glucose-1-phosphate (dipotassium salt)	100 mg
adenosine-5-phosphoric acid	10 mg
sodium fluoride	20 mg
glycogen	4 mg
acetate buffer 0.1 M pH 6.0	10 ml
polyvinyl pyrrolidone (average MW = 12,000)	2 g
ethanol	1 ml

The various components were dissolved in the buffer solution before the addition of ethanol. When stored in the refrigerator the medium was found to be stable for at least one week. The medium was heated to room temperature before use. Glucose 1 phosphate and adenosine-5-phosphoric acid were obtained from Sigma, USA, sodium fluoride and glycogen from Merck, Germany and polyvinyl pyrrolidone from Koch-Light, Great Britain.



Fig. 1. Incubation procedure. The inverted blood film is placed on top of a thick slide with a concavity which contains the incubating medium.

Histochemical Procedure

(1) A film of capillary blood (or a film of a leucocyte concentrate) was dried in air and was left for not longer than 30 min.

(2) The blood film was incubated in the incubating medium for one hour at 37 C under conditions suitable to prevent evaporation. For routine purposes a thick slide with a central shallow concavity (22 mm in diameter) was used. The concavity was filled with incubating medium, and the slide with the blood film was inverted and placed firmly on top of the thick slide (Fig. 1). Subsequently the preparation was placed in an incubator.

(3) The blood film was removed from the thick slide, dipped in 40% ethanol and dried quickly in hot air.

(4) Next, the blood film was fixed for one minute in methanol and dried in air.

(5) Before examination under the microscope a coverslip was mounted on the area of the blood film which had been in contact with the incubating medium, using a drop of glycerine-iodine (2 ml of Gram's iodine solution to 3 ml of glycerine). The resulting staining reaction was stable for several hours.

(6) When desirable the preparation was stored for later examinations after removal of the coverslip and rinsing in 40% ethanol.



Fig. 2. Phosphorylase reaction.) Neutrophils, lymphocytes and thrombocytes in leucocyte concentrate film. *b)* Neutrophil and lymphocyte in a blood film.) Basophil. *d)* Monocyte.) Eosinophil.

Results

For the purpose of describing the method we confined our study to healthy subjects. The leucocytes in the blood films were well preserved morphologically after incubation, and when the films had been mounted in an iodine-containing medium the great majority of the different types of white cells presented a blue or violet staining reaction (Fig. 2).

The *neutrophilic granulocytes* gave the strongest staining reaction, since all cells belonging to this type showed a uniform coloration of moderate intensity. All *monocytes* also gave a uniform, but weaker staining of the cytoplasm. In the majority of the *lymphocytes* the narrow cytoplasmic rim gave a weak or moderate diffuse staining reaction or contained distinct iodophilic granules, but some were completely negative. In the *eosinophilic* granulocytes the granules were negative, but the intergranular substance was stained with a moderate intensity. *Basophilic granulocytes* contained coarse intensely stained granules, probably representing the basophilic granules. The identification of this cell type was facilitated by the examination of films of leucocyte concentrates prepared by a

modification of EFRATI and ROZENSZAJN's method (1). The *thrombocytes* contained an intensely stained coarse granule probably at the site of the granulomere. The *erythrocytes* usually washed off during incubation.

Evaluation of the Method

A series of experiments were made to test the specificity of the method and the influence of some of the components of the incubating medium.

Specificity of the reaction. When glucose 1-phosphate was omitted from the incubating medium no staining was observed in any cell type, indicating that the iodophilic substances were synthesised during incubation and did not represent preformed polysaccharides. This finding is in agreement with the fact that preformed glycogen in normal blood leucocytes is not iodophilic (9). Substitution of glucose 1 phosphate by uridine diphosphate glucose also abolished the staining reaction, indicating that the polysaccharide synthesis takes place directly from glucose 1-phosphate and not via the glycogen synthetase system.

Effect of polyvinyl pyrrolidone. The effect of the addition of polyvinyl pyrrolidone was tested by preparing a series of incubating media containing different amounts of this substance. A rough estimate of the resulting staining intensity of lymphocytes, neutrophils and thrombocytes and the cellular damage incurred during incubation is recorded in Table I. It appears from this experiment that increasing amounts of polyvinyl pyrrolidone up to an optimum of 2 to 2.5 g increase the reaction by preventing damage to the leucocytes. Greater amounts inhibit the enzyme reaction.

Effect of ethanol. In similar experiments the effect of ethanol on the reaction was examined (Table II). When ethanol was omitted from the medium all lymphocytes were strongly positive containing coarse intensely stained granules, however, most neutrophils remained unstained, and only a few presented an intense staining reaction. This result is explained by the observation that most neutrophils were very badly preserved after incubation in the ethanol free medium and presumably the enzyme or the synthesised polysaccharide had leaked from the cells. The lymphocytes on the other hand were shrunken, but otherwise seemed intact. Increasing ethanol concentrations first enhanced the reaction in the neutrophils by diminishing cellular damage, but at the same time the

Table I
Effect of polyvinyl pyrrolidone on phosphorylase reaction

	g PVP added to 11 ml incubating medium						
	0	1	1,5	2	2,5	3	4
Neutrophils	0	(+)	+ to +++	+++	+++	++	0
Lymphocytes	0	(+)	+	+	+	+	0
Thrombocytes	++	+	+	+	+	+	0
Cellular damage	+++	++	+	0	0	0	0

Table II
Effect of ethanol on phosphorylase reaction

	ml ethanol added to 10 ml incubating medium					
	0	0,5	1	2	3	4
Neutrophils	0(to+++)	+ to +++	+++	++	++	+
Lymphocytes	+++	++	+	(+)	0	0
Thrombocytes	+++	++	+	+	+	+
Cellular damage	+++	+	0	0	0	0

staining of lymphocytes and thrombocytes diminished due to enzyme inhibition Addition of 1 ml ethanol to the medium constituted the optimum by giving a positive reaction in all neutrophils and in the majority of the lymphocytes

Influence of sodium fluoride and insulin Omission of sodium fluoride from the medium only weakened the reaction slightly Addition of insulin to the medium, which according to TAKEUCHI AND KURIAKI (7) enhances the reaction was in the present study found to have no effect.

Influence of pH Reduction of the pH of the incubating medium to pH 5 6 weakened the staining result, whereas equally strong staining was observed at pH 6 0 and pH 7 0 The latter experiment was performed using a tris buffer (0 1 M) These results show a higher pH optimum for the reaction than that found by TAKEUCHI AND KINOSHITA (6)

Conclusions

Previously the phosphorylase activity in blood films has been studied only after prefixation. TAKEUCHI AND KINOSHITA (6) used methanol-fixed films and demonstrated phosphorylase activity in some of the neutrophils. QUAGLINO AND HAYHOE (3) who employed acetone fixation observed phosphorylase activity in all granulocytes and also in some monocytes and lymphocytes. The

present method, which avoids enzyme destruction by prefixation, was more sensitive and in our experience more reliable. The results resembled those obtained by QUAGLINO AND HAYHOE, but phosphorylase activity was demonstrated in all white cells with the exception of some small lymphocytes. The fact that all lymphocytes possess some phosphorylase activity was however revealed by the experiments, where ethanol was excluded from the incubating medium.

The staining reaction with iodine was either blue or violet. Phosphorylase is only capable of synthesizing unbranched polysaccharide chains, which stain blue, and the observation of a violet coloration is therefore indicative of branching enzyme activity. The branching enzymes are however largely inhibited in these experiments due to the presence of ethanol (5).

The distribution of phosphorylase in the various cell types closely corresponds to the distribution of preformed glycogen, as previously recorded using the hexamine-silver technique (11). It was then shown that almost all lymphocytes also in normal subjects contain small amounts of glycogen. This correlation between the distribution of phosphorylase and glycogen is hardly surprising considering the physiological role of the enzyme, but nevertheless it is possible that preformed glycogen to some extent influences the outcome of the histochemical phosphorylase reaction by acting as a primer for polysaccharide synthesis (3).

The mode of action of polyvinyl pyrrolidone is not well established. The use of this inert substance in histochemistry was first recommended by SCARPELLI *et al.* (4), although in much smaller concentrations. It may simply protect the cells by increasing the viscosity of the medium, and it is suggested that high concentrations of polyvinyl pyrrolidone may also be of use in the haematological application of other histochemical procedures.

Summary

A new method was described for the histochemical demonstration of phosphorylase in films of peripheral blood and leucocyte concentrates. The unfixed air-dried films were protected during incubation by using an incubating medium, which contained high concentration of polyvinyl pyrrolidone. The method proved more sensitive than previously reported methods, and phosphorylase activity was found in all granulocytes and monocytes, as well as in most lymphocytes. The influence of various constituents of the medium was evaluated.

Zusammenfassung

Es wird eine neue Methode zum histochemischen Nachweis von Phosphorylase in Ausstrichen von peripherem Blut und von Leukocytenkonzentraten beschrieben. Bei der Inkubation werden die unfixierten, luftgetrockneten Ausstriche durch eine Inkubationslösung mit hoher Konzentration an Polyvinylpyrrolidon geschützt. Die Methode erwies sich als empfindlicher als früher beschriebene Verfahren und ergab den Nachweis von Phosphorylase in allen Granulocyten und Monocyten sowie in den meisten Lymphocyten. Der Einfluss verschiedener Bestandteile des Mediums wurde untersucht.

Résumé

Une nouvelle méthode histochimique pour la mise en évidence de la phosphorylase dans les frottis de sang périphérique et de leucocytes concentrés est décrite. Les frottis non-fixés et séchés à l'air sont protégés durant l'incubation par une solution ayant une concentration de polyvinyl-pyrrolidone très forte. Cette méthode se révéla être plus sensible que les procédés antérieurs et mit en évidence de la phosphorylase dans tous les granulocytes et monocytes, ainsi que dans la plupart des lymphocytes. L'influence des différents constituants de la solution a été étudiée.

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An Evaluation of Erythropoiesis in Canine Marrow*

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Although reference has been made to ineffective erythropoiesis in normal animals (4, 13), this has not yet been substantiated by any direct experimental evidence. A recent analysis of cell fluxes during granulocyte formation in the dog has revealed a considerable ineffective granulopoiesis (7, 11, 12). A similar analysis has now been made for erythropoiesis in canine marrow and the findings will be reported in this article.

Materials and Methods

Bone marrow preparations from two young adult beagles used in the analysis of granulopoiesis (7) and similar preparations from another beagle were used in this study. Proerythroblasts, basophilic normoblasts and early polychromatic normoblasts were included in the proliferative cell class (P); these cells are flash-labeled with tritiated thymidine (H^3T). Late polychromatic normoblasts and orthochromatic normoblasts with distinct nuclear chromatin were grouped in the nonproliferative class (NP) since it was not known definitely whether transition from P after the last division occurred only to the late polychromatic cell or to the orthochromatic cell as well; the frequency of the latter is small in canine marrow. At least 1000 NP and all associated P, intact red cells with pyknotic nuclei (CPN) and free pyknotic red cell nuclei (FPN) were scored at each interval for each dog. A minimum of 100 erythroid mitoses (metaphases and anaphases) were scored for the labeled mitosis study. Strong cytoplasmic basophilia was used as the criterion to distinguish erythroid from myeloid mitoses in the case of large cells, whereas small mitotic figures presented no difficulty in the Giemsa-stained preparations. Any cell with 2 or more silver grains over the nucleus was scored as labeled.

Results and Discussion

Figure 1 shows the percent labeled erythroid metaphases and anaphases in marrow (average for 3 dogs) with time after injection of H^3 thymidine. The pattern was the same whether mitoses were grouped for the whole erythroid series (Fig. 1) or classified in terms

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Table I
Cell fluxes during erythroid development.

	Pro-erythroblast	Basophilic normoblast	Early polychromatic normoblast	NP	CPN + TPN
Relative Number (N)	25	106	359	465	71
0.5 hour labeling index (N_s /N)	0.65	0.56	0.39	0	0
Number in DNA synthesis (N_s)	16.3	59 4	140	0	0
Birth rate (K_p)	2.8	10.1	23.7	0	0
Influx (K_I)	+	2.8	12.9	37.2	—
Efflux (K_e)	2.8	12.9	36.6	—	—
Mean No. of mitosis	—	2.3	1 4	—	—

$K_p = N$ /T wher T = 3.9 h.
Indicates an unknown stem cell efflux into the procrythroblast pool; this is probably very small compared to the succeeding fluxes.
Determined from a turnover rate of 8%/h.
$K_e = K_I + K_p$
$K_o/2K_I$

Table II
Actual and theoretical recovery of labeled nonproliferative cells.

	Early polychromatic normoblast	Late polychromatic and orthochromatic normoblast (nonpyknotic)
Relative Population Number	359	465
Percent labeled	28.5 (0.5 h 4+gr)	33.8 (7.5 h 2+gr)
Number labeled	102	157

which would swing the balance toward underproduction rather than overproduction or ineffective production.

A comparison of the actual recovery and the theoretical recovery into NP of early polychromatic cells after one division provides additional evidence for the absence of ineffective production. The cohort of 4+ cells in S in the last generation should be seen as 2+ cells in NP theoretically at a time given by s + t or 7.5 h, where s is the interval occupied by 4+ cells in S (5 1 h) and t is the time from the end of s to recognition of these cells in NP (2.4 h) From the flux relationships in Table I the mean number of divisions occurring in the early polychromatic stage is 1 4 In that case after one division one labeled early polychromatic cell should give rise to 1.56 cells in the NP compartment 102 of the former cells should give rise to 159 of the latter cells, and as shown in Table II the actual recovery was 157 Thus, ineffective erythro-

poiesis seems to be absent or insignificant in dog marrow up to the late polychromatic and orthochromatic stage. In regard to subsequent stages, an assessment of possible attrition before entry in blood can be made by comparing the daily production in marrow with the daily turnover in blood. If the approximate total nucleated erythroid population in dog marrow is 7×10^9/kg (2) total production can be estimated as 6×10^9/kg/day from the H³T labeling index and the S period. This value when compared with a daily turnover of 4.8×10^9/kg/day in the peripheral blood (10) reveals an imbalance, suggestive of an ineffective production of some 20% of total production. If this crude approximation is representative of the true picture we would infer that there is some loss after the orthochromatic stage, e. g. during the loss of nucleus and transition to a reticulocyte or in the reticulocyte stage itself.

Summary

Erythropoiesis in canine marrow was evaluated by an analysis of cell fluxes among the erythroid components after single injection of tritiated thymidine in 3 dogs. Erythroid precursors had an average cell cycle time of about 10 h which could be subdivided into the following time components on average: pre-DNA synthesis ~2 h; DNA synthesis ~6 h, post-DNA synthesis ~1 h; mitosis ~1 h. The average age of nondividing red cell at the beginning of nuclear pyknosis was ~12.5 h, there was a wide age distribution with minimum age of 6 h. No ineffective production was detected up to the orthochromatic stage before nuclear pyknosis. A crude comparison of the total daily production in marrow with the daily turnover in blood is suggestive of 20% cell loss between the process of enucleation and entry into circulation.

Zusammenfassung

Bei 3 Hunden wurde die Erythropoese untersucht durch Bestimmung der Zellverschiebungen unter den Komponenten der Erythropoese nach einer einmaligen Injektion von Tritium-Thymidin. Die roten Vorstufen wiesen eine mittlere Dauer ihres Zyklus von 10 Stunden auf. Diese ließ sich unterteilen in folgende mittlere Abschnitte: vor der DNA-Synthese ~2 Std., DNA-Synthese ~6 Std., nach der DNA-Synthese ~1 Std., Mitose ~1 Std. Das mittlere Alter einer nicht in Teilung befindlichen roten Zelle zu Beginn der Kernpyknose betrug ~12,5 Std. Es fand sich eine breite Streuung des Alters mit einem Minimum von 6 Std. Bis zum orthochromatischen Stadium vor der Kernpyknose war keine ineffektive Produktion festzustellen. Ein grober Vergleich der gesamten täglichen Produktion im Knochenmark mit dem täglichen Umsatz im Blut spricht für einen Zellverlust von 20% zwischen dem Prozeß der Entkernung und dem Übertritt in die Zirkulation.

Résumé

L'érythropoïèse de la moelle osseuse du chien a été évaluée à l'aide de l'analyse du flux des cellules parmi les composantes érythroïdes après une injection unique de thymidine tritiée à 3 chiens. Les précurseurs érythroïdes ont un cycle cellulaire d'environ

10 heures, temps qui peut être subdivisé dans les composantes moyennes suivantes: synthèse pré-ADN ~2 h; synthèse de l'ADN ~6 h; synthèse post-ADN ~1 h; mitose ~1 h. L'âge moyen des érythrocytes ne se divisant pas au début de la pyknose nucléaire était de ~12,5 h; les variations suivant l'âge sont très grandes, le minimum est de 6 h. Aucune production inefficace ne put être décelée jusqu'au stade orthochromatique avant la pyknose nucléaire. Une comparaison grossière de la production journalière dans la moelle osseuse avec le turnover journalier dans le sang semble indiquer une perte de 20% des cellules entre le processus d'énucléation et l'entrée en circulation.

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P.-I. Brånemark: **Symposium on Microvascular Methodology.** Bibl. anat., fasc. 5. Karger, Basel/New York 1965. IV + 100 S., 16 Abb., 5 Tab., Preis sFr./DM 21.
Symposium anläßlich der 3. Europäischen Konferenz über Mikrozirkulation.

Wayland (California Institute of Technology) gibt einen ausgezeichneten Überblick über Definition und Bedeutung rheologischer Parameter: Viskosität homo- und heterogener Flüssigkeiten, laminäre und turbulente Strömung, Bedeutung der Reynoldschen Zahl – ein Überblick, den jedermann lesen sollte, der das Wort «Hämodynamik» häufig braucht. Klar werden die Verfahren besprochen, mit denen sich diese Parameter erfassen lassen. Besonderes Interesse verdienen die Ausführungen über die Viskosimetrie.

Brånemark und Harders befassen sich mit den Möglichkeiten der experimentellen und klinischen Bio-Mikroskopie. Während die Technik der üblichen histologischen Verarbeitung für das Studium der Gefäßarchitektur durch Wollnow besprochen wird, fehlt leider ein Beitrag über die Leistungsfähigkeit der Elektronenmikroskopie. [illegible] berichtet über die Möglichkeiten intra- und extravasculärer Kapillar-Druckmessung. Methoden und Ergebnisse zur Prüfung der Kapillarpermeabilität (Kantharidenblase und vitale Studien mit Farbstoffen und Fluorescein) werden von Witte und Schwartzkopf besprochen. Moreno bietet eine knappe Übersicht über Methoden zur Messung von korpuskulärer Deformation, Strömungsgeschwindigkeit und Gefäßdurchmesser.

Das sorgfältig redigierte Buch vermittelt eine gute Übersicht über den heutigen Stand und die zukünftigen Wege der Forschung in der Mikrozirkulation. Dank der Qualität der Referenten und der zahlreichen Literaturzitate ist es auch für jeden wertvoll, der sich mit einem Teilproblem der Mikrozirkulation befassen will. Kliniker mit hämodynamischen Neigungen seien besonders auf die grundlegenden Beiträge von Wayland hingewiesen.

L. K. Widmer, Basel

Donald G. McKay: **Disseminated Intravascular Coagulation.** Hoeber Medical Division, Harper & Row, New York/London 1965. 493 pp., 204 Illustr. Price $ 16.

The author considers disseminated intravascular coagulation as a fundamental mechanism in many diseases. After a short review on blood coagulation the main part of the book discusses intravascular coagulation in a wide variety of clinical syndromes, among these e.g. sickle cell anemia, Paroxysmal nocturnal hemoglobinuria, acquired hemolytic anemia. In reading the book one has the impression that the importance of intravascular coagulation for the pathogenesis of a wide variety of diseases and syndroms is somewhat overrated.

O. Rosnow, New York

Shirley A. Johnson and Tibor J. Greenwalt: **Coagulation and Transfusion in Clinical Medicine.** Little, Brown and Co., Boston 1965. 203 pp. Price $ 9.50.

This little book is intended as a guide for the general practitioner and residents (assistants) in a hospital for the management of bleeding conditions. The authors have coordinated their respective experience in management of a blood bank and clinical hematology. A short introductory chapter on blood coagulation is followed by an outline of transfusion therapy. Chapters on hemophilia, thrombocytopenia, anticoagulants, coagulation in liver disease, coagulation mechanism in the newborn are given. The book is a kind of primer for the uninitiated or to fresh up on the subject. It contains a fairly long list of references.

G. Rosnow, New York

H. Koblet, P. Vesin, H. Diggelmann and S. Barandun. **Physiology and Pathophysiology of Plasma Protein Metabolism.** Proceedings of the Third Symposium, held at Grindelwald, September 10–12 1964. Verlag H. Huber Bern 1965. 240 S., 79 Abb., 28 Tab. Preis sF /DM 27.–

Im August 1961 diskutierte im Hôpital St. Antoine in Paris eine Gruppe jüngerer Kliniker die Problematik des gastrointestinalen Eiweissverlustes. Im Vordergrund des Interesses standen damals die mit dem Gordon-Test erhaltenen Befunde. Dieselbe Gruppe traf sich 1963 in Brügge, 1964 tagte sie in Grindelwald. Dabei ist es interessant zu sehen, wie sich in Kurze ihr Interessenbereich weit uber die ursprungliche Fragestellung hinaus entwickelt hat. So standen anlässlich der *Grindelwaldner* Tagung zahlreiche Aspekte der Physiologie und Pathologie des menschlichen Plasmaeiweiss-Stoffwechsels zur Diskussion, wobei zu Recht methodologische Fragen einen breiten Raum einnahmen. Die vorliegende Monographie vermittelt einen ausgezeichneten Überblick über ein aktuelles Gebiet biochemischer und klinischer Forschung. A. Hässig, Bern

Warren Andrew. **Comparative Hematology.** Grune & Stratton Inc., New York/London 1965. 188 pp. *Price* $ 22.75.

This well printed and illustrated large sized book is mainly a descriptive presentation of the morphology and particularly the histology of the blood forming organs of the invertebrates and vertebrates. Comparative aspects of the blood of invertebrates, discussed in a separate chapter include remarks about the clotting mechanism; unfortunately the information about the role of the blood in defense reactions is very scanty. The part on the vertebrates comprises the development of hematopoietic tissue in pisces, reptils, birds and mammals. A number of electron microscope illustrations of blood cells are reproduced in color. this section, particularly of the peripheral blood cells contains also the excellent color plates from the Atlas of Avian Hematology by LUCAS AND JAMROZ. A more comprehensive comparative presentation of the hematology of invertebrates and vertebrates would have been welcome. The role of the reticulo-endothelial system in mammals and its behavior in invertebrates is barely scratched. The section on diseases of the blood in man and animals is limited to a few comments.

The book, although falling short of a comparative hematology in the proper sense, contains many facts and data which may be of importance for the human hematologist too. O. Rosenow, New York

Acta haemat. 35: 321-332 (1966)

Department of Paediatrics, University of Rome (Chief: Prof. A. Colarizi)

Contribution to the Knowledge of the Immunological Properties of Adult and Foetal Haemoglobin and of Their Polypeptide Chains

G. Maggioni, C. Ioppolo and G. Gerlini

It has long been known that the human haemoglobin possesses antigen properties, but only with the progress of the techniques of separating the haemoglobins it has been possible to demonstrate the antigen specificity of the normal haemoglobins HbA_1, HbA_2 (1, 2, 8, 19) HbF (1, 2, 5, 6, 7, 8, 22) and of the pathological ones HbS, HbC, Hb Bart's (2, 19), Hb Lepore (2) and to commence the study of the antigen properties of the polypeptide chains constituting the haemoglobins.

In 1963 Maggioni *et al.* (17) put forward the hypothesis that the antigenic specificity of HbF resides in the γ chain; in 1964 Boerma and Huisman (2) affirmed that the α, β, γ and δ chains all possess antigen capacities and demonstrated several antigenic sites, some of them in common; in the same year Rose *et al.* (19) observed that the β, γ and δ chains are the most active antigen sites of haemoglobins A, A_2, F and Bart's and that in them reside the common antigenic groups of the haemoglobins themselves.

Boerma and Huisman and Rose *et al.* deduced the antigen properties of the various polypeptide chains by using in their immunodiffusion and absorption studies human haemoglobins differing in one of the two pairs of polypeptide chains.

Recently Yakulis and Heller (23) and Kleihauer and Milenyi (12a) using immunological and physico-chemical techniques respectively on hybrids of HbF with dog haemoglobin, affirmed that the immunological, physico-chemical and functional characteristics of HbF can be basically referred to the γ chain.

In the present paper we report on the antigen properties of the polypeptide chains of adult and foetal haemoglobin separated by

means of hybridization with dog Hb The dog haemoglobin, which furnished the globinic subunity of the hybrids, has never reacted with our antisera.

Material and Methods

Preparation of the Antigens

Foetal haemoglobin. The HbF was prepared from umbilical cord blood of group 0 Rh positive after washing and haemolysis of the erythrocytes according to current methods and by means of alkali denaturation according to the method of Singer *et al.* (21) The alkali resistant fraction was then purified on block of starch according to Kunkel (13) eluted and sterilized by passing through Seitz filters. The samples used for the immunization, each containing 20–40 mg of haemoglobin, were mixed with an equal volume of complete Freund adjuvant and kept in the refrigerator

Adult haemoglobin was prepared from red corpuscles of normal adults of group 0 Rh positive, washed and haemolyzed. The greater fraction of the normal adult haemoglobin was prepared on block of starch according to Kunkel' method, eluted and sterilized through Seitz filters. The samples used for the immunization were prepared in the same way as for the HbF

Both the adult and the foetal haemoglobin were used in the form of carboxyhaemoglobin.

HbF and HbA hybrids. The hybrids of HbA and HbF with dog haemoglobin were prepared according to Itano method (12) already experimented with in our laboratory by Maggioni *et al.* (15) and by Bottini *et al.* (4). The hybrids obtained were prepared on block of starch according to Kunkel (13) and eluted. In this system three bands appear: the first, nearest the anode, is formed of the hybrid $\alpha^{can}\beta^{A}$ and respectively $\alpha^{can}\gamma^{F}$ the slow one, nearest the cathode, is represented by the hybrid $\alpha^{A}\beta^{can}$ while the third, intermediate band consists of the haemoglobins set out with.

Immunization of the rabbits. Six rabbits were immunized, 3 with HbA and 3 with HbF with total of 8 injections at distance of 10 days from each other the first dose was 40 mg, the successive doses 20 mg each. At distance of 6 days from the last inoculation, the blood was taken for preparing the anti-serum.

Immunodiffusion. The antigen-antibody reactions were carried out with the double diffusion method in agar gel according to Ouchterlony (18) modified by [illegible] *et al.* (1)

Absorptions. The rabbit antisera were absorbed with the entire haemoglobins used for the immunization and with the respective hybrids until the disappearance of the reaction with the homologous antigen.

Results

The serum of the rabbits immunized with HbF reacted to the immunodiffusion both with HbF and the respective hybrids and with HbA and the respective hybrids. The reaction with HbF was performed at concentrations between 1 and 0 06 g / Put in contact with HbF at a concentration of 0.2 g / which has proved to be the optimal concentration for the antigen antibody reaction, the serum gave an antibody titre of 1 4 The same antibody titre was obtained

with the HbF fast hybrid at a concentration of 0.2 g %. With regard to the HbF slow hybrid, the HbA and the respective slow and fast hybrids, the reaction disappeared with the dilution of the serum 1 2

With the entire HbA as antigen, a reaction occured at concentrations between 1 and 0 2 g %.

From the qualitative analysis of the reactions between the antiserum and the various antigens, taken at various concentrations, it will be seen that the reaction with HbF is of an intensity equal to that with the HbF fast hybrid, while this reaction becomes progressively weaker when the antiserum is put in contact with the HbF slow hybrid, with HbA and the slow and fast hybrids of this (Table I Fig 1)

Still examined at the point of equivalence with respect to the antiserum being studied, the HbF gives an identity reaction with the respective fast hybrid (Fig 1) while the reaction with the HbF slow hybrid, the HbA and the hybrids of this, is of partial identity with pictures of greater antigenic complexity on the part of the HbF (Fig 1 2, 3)

The HbF fast hybrid further presents a behaviour identical with HbF when it is compared with HbA and the hybrid of this (Fig 2, 3 4) Because of the weak reaction of the HbA and its hybrids with the antiserum being studied, it is difficult to interpret the morphology of the respective lines of precipitation the HbA forms two short thin lines which give an identity reaction with the HbA fast hybrid for one of the two lines (Fig 1)

Table I
Reactions of antiserum anti-HbF during the immunodiffusion test.

Antigens	Non absorbed serum	Serum absorbed with HbA	HbF	α β^{can}	α^{F} β^{can}	α^{can} β^{A}	α^{can} γ^{F}
HbA	+	—	—	±	±	±	—
HbF	+++	+++	—	++	++	++	—
SA	+	—	—	—	—	—	—
SF	++	±	—	—	—	+	—
FA	+	—	—	±	±	—	—
FF	+++	++	—	++	++	++	—

SA slow HbA hybrid (β^{can})

SF slow HbF hybrid (α^{F} β^{can})

FA fast HbA hybrid (α^{can} β)

FF fast HbF hybrid (α^{can} ^{F})

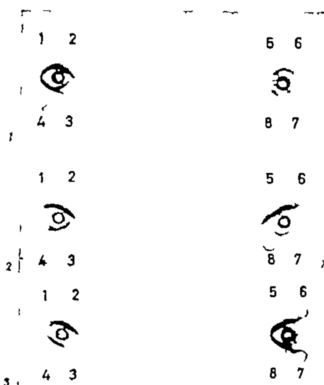


Fig. 1. 1 = HbF, 2 = $\alpha^{can}\gamma^{F}$, 3 = $\alpha^{F}\beta^{can}$, 4 = HbF, 5 = HbA, 6 = $\alpha^{A}\beta^{can}$, 7 = $\alpha_{s}^{can}\beta^{A}$, 8 = HbA, in the middle anti-HbF

Fig. 2. 1 = HbA, 2 = $\alpha_{s}^{can}\gamma^{F}$, 3 = $\alpha^{F}\beta^{can}$, 4 = HbA, 5 = HbF, 6 = HbA, 7 = $\alpha^{can}\beta^{A}$, 8 = $\alpha^{A}\beta^{can}$, in the middle anti-HbF

Fig. 3. 1 = $\alpha^{A}\beta^{can}$, 2 = $\alpha_{s}^{can}\gamma^{F}$, 3 = $\alpha_{s}^{can}\beta^{A}$, 4 = $\alpha_{s}^{F}\beta^{can}$, 5 = HbF, 6 = $^{A}\beta^{can}$, 7 = $^{can}\beta^{A}$, 8 = HbF, in the middle anti-HbF

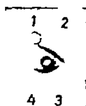


Fig. 4. 1 = $\alpha^{can}\beta^{A}$, 2 = $\alpha^{can}\gamma^{F}$, 3 = $\alpha_{s}^{F}\beta^{can}$, 4 = $\alpha^{A}\beta^{can}$, in the middle anti-HbF

The HbF slow hybrid gives an identity reaction with that of the HbA (Fig. 4), while both furnish a picture of partial identity with the HbA fast hybrid with a picture of greater antigenic complexity in favour of the slow hybrids (Fig. 3).

Absorption When the antiserum being studied is absorbed indifferently with the HbF and its fast hybrid, all the reactions disappear both towards the entire haemoglobins and towards their hybrids. When it is absorbed with the slow hybrid of both the HbF and the HbA, the reaction with them disappears that with the HbA and its fast hybrid appears scarcely perceptible, while the reaction with the HbF and its fast hybrid remains quite clear (Fig 5) When it is absorbed with HbA, the reaction with this and its hybrids disappears and a serum remains which reacts clearly with the HbF less intensely with the HbF fast hybrid and very weakly with the HbF slow hybrid (Fig 13)

The absorption with the HbA fast hybrid caused the persistence of the reaction with HbF and its hybrids, while the reaction with the HbA is not only very slight but sometimes, if the reaction conditions are varied, is not perceptible (Fig 5) the reaction with the HbA slow hybrid, on the contrary disappears in every case.

The serum of the rabbits immunized with HbA reacted to the immunodiffusion both with HbA and its respective hybrids and with HbF and its respective hybrids (Table II Fig 7 8) The reaction with the HbA occured at concentrations between 1 and 0.2 g %. Put in contact with HbA at a concentration of 0.2 g %, which in this case also proved optimal for the antigen antibody reaction, the serum gave an antibody titre of 1 4 With the HbF as antigen a reaction was obtained with the haemoglobin at concentrations between 1 and 0 06 g %. The antibody titre both towards the HbA and the HbF hybrids resulted 1 2.

At the immunodiffusion the HbA and HbF give a cross reaction picture (Fig 6) which is repeated when the HbA is compared with the HbF fast hybrid (Fig 7) and the HbA and HbF fast hybrids are compared with each other (Fig 8)

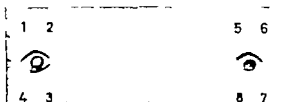


Fig 5. 1 HbF 2 = $\alpha^{can}\gamma^{F}$ 3 = $\alpha^{can}\gamma^{F}$ 4 = HbA, in the middle anti-HbF absorbed with $\alpha^{A}\beta^{can}$ 5 = HbF 6 = $\alpha_2^{can}\gamma^{F}$ 7 = $\alpha_2^{can}\beta$ 8 = HbA, in the middle anti-HbF absorbed with $\alpha_2^{can}\beta$

Table II
Reactions of antiserum anti HbA during the immunodiffusion test.

Antigens	Non absorbed serum	Serum absorbed with HbA	HbF	$\alpha_s^A \beta^{can}$	$\alpha_s^F \beta^{can}$	$\alpha^{can} \beta^A$	$\alpha^{can} \gamma^F$
HbA	+++	—	++	++	++	—	—
HBF	+++	++	—	++	++	++	—
SA	++	—	—	—	—	—	—
SF	++	—	—	—	—	—	—
FA	++	—	±	±	±	—	±
FF	++	++	—	±	±	±	—

SA slow HbA hybrid ($\alpha_s^A \beta^{can}$)

SF slow HbF hybrid ($\alpha_s^F \beta^{can}$)

FA fast HBA hybrid ($\alpha^{can} \beta^A$)

FF fast HbF hybrid ($\alpha^{can} \gamma^F$)

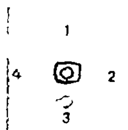


Fig. 6. 1 = HbF 2 = HbA, 3 = HbF 4 = HbA, in the middle anti HbA.



Fig. 7. 1 = $\alpha_s^F \beta^{can}$ 2 = HbA, 3 = HbF 4 = $\alpha^F \beta^{can}$ 5 = $\alpha^{can} \gamma^F$ 6 HbA, 7 = HbF 8 = $\alpha^{can} \gamma^F$ in the middle anti-HbA.

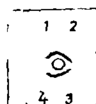


Fig. 8. 1 = $\alpha_s^A \beta^{can}$ 2 = $\alpha^{can} \gamma^F$ 3 = $\alpha^{can} \gamma^F$ 4 $\alpha^{can} \beta^A$ in the middle anti-HbA.

The HbA compared with the HbA slow hybrid and with the HbF itself shows a partial identity reaction with a picture of greater antigenic complexity in favour of the Hb (Fig 7 9) with regard to *the fast HbA hybrid, on the contrary* an identity reaction is observed (Fig 9)

In the comparative reactions between HbF and the HbA and HbF slow hybrids respectively a picture will be seen of partial identity between the haemoglobin and the slow hybrids with an appearance of greater antigenic complexity in favour of the entire haemoglobin (Fig 7 9) The HbF on the contrary gives an identity reaction with its own fast hybrid (Fig 7)

Absorption. When the antiserum being studied was absorbed with HbF the disappearance was obtained of the reaction with this, with the HbF fast hybrid and the HbA and HbF slow hybrid respectively while the reaction is still observed with the HbA and its respective fast hybrid (Fig 14) Absorption with the HbF fast hybrid gave rise to an antiserum which behaved in exactly the same way as the antiserum absorbed with HbF (Fig 16) When absorbing with HbA, the disappearance was observed of the reaction with this, with the HbA fast hybrid and both the HbA and HbF slow hybrids, while the reaction with the HbF and its fast hybrid persisted (Fig 15) In this case too absorption with the HbA fast hybrid gave rise to an antiserum which reacted in the same way as the antiserum absorbed with HbA (Fig 16)

When absorbing both with the HbA slow hybrid and the HbF slow hybrid, the disappearance of the reaction with these hybrids was obtained the reaction with the entire HbA and HbF persisted very clearly although slightly weaker than that of the non-absorbed serum still weaker but clear was the reaction with the HbA and HbF fast hybrids.

The lines of precipitation obtained in the various immunodiffusion tests always gave a distinct colouring with benzidine, demonstrating the haemoglobinic nature of the immunoprecipitates. The dog haemoglobins, cemented at various concentrations with our antisera, never showed precipitation reactions.

Discussion

The anti HbF serum reacted both with the HbF and its hybrids and with the HbA and its respective hybrids (Fig 1) This behaviour could be attributed to the presence in the antiserum being studied

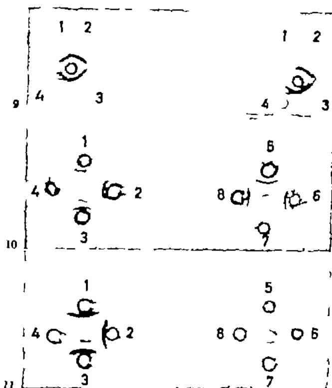


Fig. 9 1 = $\alpha_2^A \beta^{can}$ 2 = HbA, 3 = HbF 4 = $\alpha^A \beta^{can}$ 5 = $\alpha_2^{can} \beta^A$ 6 = HbA 7 = HbF 8 = $\alpha^{can} \beta^A$ in the middle anti-HbA.

Fig. 10 1 = anti-HbA absorbed with $\alpha_2^A \beta^{can}$ 2 = anti-HbA absorbed with $\alpha^F \beta^{can}$ 3 = anti-HbA absorbed with $\alpha_2^{can} \beta^A$ 4 = anti-HbA absorbed with $\alpha_2^{can} \gamma^F$ in the middle HbF 5 = anti-HbA absorbed with $\alpha_2^A \beta^{can}$ 6 = anti-HbA absorbed with $\alpha^F \beta^{can}$ 7 = anti-HbA absorbed with $\alpha^{can} \beta^A$ 8 = anti-HbA absorbed with $\alpha_2^{can} \gamma^F$ in the middle HbA.

Fig. 11 1 = anti-HbF absorbed with $^A \beta^{can}$ 2 = anti-HbF absorbed with $\alpha^F \beta^{can}$ 3 = anti-HbF absorbed with $\alpha^{can} \beta^A$ 4 = anti-HbF absorbed with $\alpha^{can} \gamma^F$ in the middle HbF 5 = anti-HbF absorbed with $\alpha^A \beta^{can}$ 6 = anti-HbF absorbed with $\alpha_2^F \beta^{can}$ 7 = anti-HbF absorbed with $\alpha_2^{can} \beta^A$ 8 = anti-HbF absorbed with $\alpha_2^{can} \gamma^F$ in the middle HbA.

of several specific antibodies or to the antigenic resemblance between the haemoglobins and their respective polypeptidic chains. The latter hypothesis seems more probable.

In fact, the antibody titre of the serum being studied towards the HbF is higher than that towards the HbA at the immunodiffusion the HbF presents a picture of greater antigenic complexity

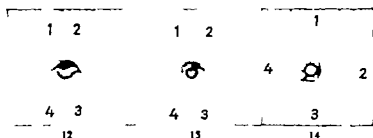


Fig 12. 1 = $\alpha_2^F \beta^{can}$ 2 = $\alpha^{can} \gamma^F$ 3 = $\alpha^{can} \beta^A$ 4 = $\alpha^A \beta^{can}$ In the middle anti-HbF

Fig 13. 1 = $\alpha_2^F \beta^{can}$ 2 = $\alpha^{can} \gamma^F$ 3 = $\alpha^{can} \beta^A$ 4 = $\alpha_2 \beta^{can}$ In the middle anti-HbF absorbed with HbA.

Fig 14 1 = HbF 2 = HbA, 3 = $\alpha_2^{can} \beta$ 4 = $\alpha_2^{can} \gamma^F$ In the middle anti-HbA ab-sorbed with HbF

than the HbA when the antiserum is absorbed with HbF every reaction disappears, while when it is absorbed with HbA the reaction of the antiserum with the HbF remains (Fig 13)

Furthermore, it seems possible that the anti HbF antibodies can be identified with the anti γ antibodies. In fact the HbF and its fast hybrid show an identity reaction at the immunodiffusion (Fig 1) the antibody titre of the antiserum is the same for both, and lastly when absorbing with the HbF fast hybrid the reaction with the HbF itself disappears (Fig 11) The γ and β chains react with the antiserum since they possess antigenic determinants in common with the γ chain, as is shown by the partial identity pictures at the immunodiffusion and by the absorption tests (Fig 1 3 4 12) The α chains possess antigenic determinants in common with the γ chain and in a larger number than the β chain with which they give a partial identity reaction with a picture of greater antigenic complexity in favour of the α chains (Fig 2 3)

Also in the case of *immunization with HbA* an antiserum was obtained which reacted both with the HbA and its hybrids and with the HbF and its respective hybrids (Fig 7 8) However the behaviour of the antiserum is such as to suggest the presence in it of distinct anti HbA and anti HbF antibodies. In fact, the reaction of the antiserum with the two haemoglobins was of equal intensity at the immunodiffusion the two antigens gave a picture of partial identity with double crossing (Fig 6) Finally when the antiserum being studied was absorbed with the HbA a specific anti HbF and anti-γ antiserum was obtained (HbF fast

hybrid, Fig 15) while when it was absorbed with HbF a specific anti HbA and anti-β serum was obtained (HbA fast hybrid, Fig 14)

Moreover the serum absorbed with the HbA fast hybrid behaves in the same way as the serum absorbed with the HbA, which suggests that the antigenic specificity of the HbA is due to its β chain (Fig 10 15 16) The absorption with the HbF fast hybrid further confirms that the antigenic specificity of the HbF is due to the γ chain (Fig 16)

As regards the *behaviour of the a chains* we observe that anti-α antibodies are present both in the anti HbF antiserum and in the anti HbA antiserum, as is shown by the absorption tests with the α chains (Table I) These antibodies, however are not specific, but in our opinion are produced by antigenic determinants in common *both with the γ chain and the β chain. In fact, we have never been* able to obtain a specific anti-α antiserum.

We consider that the presence of anti HbF antibodies in the serum of the rabbits immunized with HbA should be attributed to the presence of traces of HbF in the haemolysate of the adult haemoglobin used for the immunization

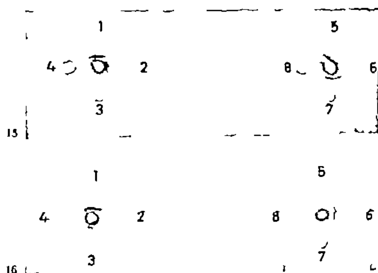


Fig 15. 1 = HbF 2 = $\alpha^{com}\gamma^{F}$ 3 = $\alpha^{com}\beta^{A}$ 4 = $\alpha^{F}\beta^{com}$ 5 = HbA 6 β^{com} 7 = HbF 8 = $\alpha^{com}\beta$ in the middle anti-HbA absorbed with HbA

Fig 16. 1 = HbF 2 = HbA 3 $\alpha^{com}\gamma^{F}$ 4 = $\alpha^{A}\beta^{com}$ in the middle anti-HbA absorbed with $\alpha^{com}\beta^{A}$ 5 = HbF 6 = HbA 7 $\alpha^{com}\beta$ 8 $\alpha^{A}\beta^{com}$ in the middle anti-HbA absorbed with $\alpha^{com}\gamma^{F}$

Summary

Both HbA and HbF possess antigenic individuality in the rabbit. The antigenic specificity of the HbA resides in the β chain, that of the HbF in the γ chain. The α chains are immunologically active for the antigenic determinants in common with the β and γ chains. The β and γ chains possess at least one antigenic determinant in common and at least one specific determinant of their own.

Zusammenfassung

Beim Kaninchen besitzen HbA als auch HbF eine Antigenspezifität. Diese befindet sich beim HbA in der β-Kette, beim HbF in der γ-Kette. Die α-Ketten sind immunologisch aktiv gegenüber den mit den β- und γ-Ketten gemeinsamen Antigenlokalisierungen. Die β- und γ-Ketten weisen mindestens eine gemeinsame Antigenlokalisierung und eine ihnen eigene spezifische Antigenlokalisierung auf.

Résumé

Aussi bien l'Hb A que l'HbF possèdent chez le lapin une individualité antigénique. La spécificité antigénique de l'HbA est située dans la chaîne beta, celle de l'HbF dans la chaîne gamma. Les chaînes alpha sont immunologiquement actives en ce qui concerne les déterminantes antigéniques qu'elles ont en commun avec les chaînes gamma. Les chaînes beta et gamma possèdent au moins une localisation commune d'antigènes et une localisation d'antigènes propre à elles-même.

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Red Cell Osmotic Fragility as an Indication of Marrow Depression after Irradiation and Effectiveness of Marrow Graft

S. M. Lewis,* I. Bleiberg and D. Danon

A rapid micromethod for the determination and automatic recording of the red-cell osmotic fragility has recently been described (2). For this a continuous reduction in salt concentration is achieved by dialysis; the 'Fragiligraph' is an instrument based on this method and devised for the simultaneous recording of cumulative and derivative fragiligrams (osmotic fragility curves). Each test requires 0.05 ml of a 1 : 30 dilution of whole blood, and the resulting fragiligrams are determined by about 100 to 150 recorded points for the derivative curve and 200 to 300 recorded points for the cumulative curve. This high resolution is of special significance in detecting, within a heterogeneous cell population, a minor group of cells which are osmotically more fragile or more resistant (3, 4). The present paper reports on the use of this method for the demonstration of variations in the red-cell population induced by irradiation and the effectiveness of marrow transplant in restoring erythropoiesis. Failure of erythropoiesis after irradiation should result in an increasing proportion of cells of more uniform age distribution. Conversely, reactivation of erythropoiesis by marrow transplant should be demonstrated by the introduction into circulation of a new population of osmotically more resistant cells. This has been borne out by the experimental results of this study.

Material and Methods

Nine male mice F (C3H×C57BL) about 3 months old and weighing about 27 g. were used in each of four similar experiments. Three of the mice were maintained untreated as controls; the remainder in lucite containers were exposed to a dose of

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1 In receipt of Wellcome Trust Research Travel Grant.

850 total body X-irradiation using General Electric Maximar III X-Ray machine (250 kv 15 ma) with 0.5 mm Cu and 1 mm Al filters, t target distance t 50 cm (dose rate, 65 /min) Immediately after irradiation 3 of the mice were injected intravenously with 3×10^6 cells from isologous bone marrow In one of the experiments 4th group was added, in which the marrow was transplanted 24 h after irradiation.

At 2 or 3 day intervals during period of 12 to 20 days 0.1 ml of blood obtained from section in the tail-tip of each mouse was collected into heparinized capillaries, of the type used in microhaematocrit determinations. Bleeding was controlled by tourniquet and in no instance did haemorrhage occur

Immediately after collection, the blood samples were diluted 1:30 in isotonic NaCl buffered to pH 7.3 with veronal cetate buffer About 0.05 ml of the blood suspension was introduced into container cell having two walls made of dialysing membrane mounted on specially constructed stainless steel frame, 0.5 mm thick. Distilled water was used as the external medium. The test was repeated several times in succession on each blood suspension. The cumulative and derivative fragiligrams were simultaneously recorded by means of the Fragiligraph Model D 2 (Elron Electronic Industries, Haifa, Israel)

Results

In the control series a normal fragiligram was obtained and this did not vary throughout the period of study (Figure, A) The sigmoidal curve depicts the cumulative fragiligram The angle and slope of the curve before reaching the plateau is governed by the time taken for completion of haemolysis, and thus by the proportion of more resistant cells which are present. The normal distribution of cells as a single population in terms of osmotic fragility is well illustrated in the unimodal derivative curve.

The irradiated animals showed the same normal fragiligram before and one day after irradiation, but an altered pattern began to appear thereafter and was clearly recognizable on the 6th day following total body irradiation (Figure B6) On subsequent days, until the mice died about 12 days after irradiation, the cumulative curve showed a progressive disappearance of the slope towards the plateau with a less rounded angle to the top shoulder of the curve and there was a narrower peak in the derivative curve. These features indicate a more uniform population with disappearance of the most resistant cells. It will be noted that on days 6 and 8 (Figure B) the base line of the cumulative curve was elevated. As the dilution of blood is constant (1 30) and the zero of the Fragiligraph recorder is aligned with no light transmission, the base line is determined by the number of cells per unit volume Thus, elevation of the base line in the cumulative curve indicates a progressively decreasing haematocrit

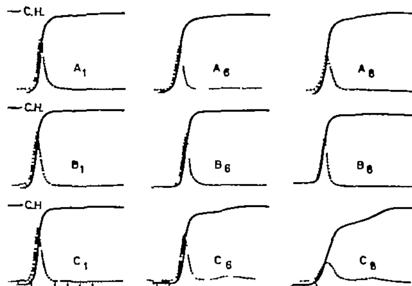


Figure. Automatically-recorded cumulative and derivative curves in representative experiment. Salt concentration on abscissa and degree of lysis on ordinate. *CH* complete haemolysis. *A* control mouse. *B* irradiated mouse. *C* mouse irradiated and subsequently injected with isologous marrow. The numbers refer to the day after the beginning of the experiment.

In the mice which were transplanted with marrow the pattern was similar to that in the irradiated mice for the first few days with slight individual variations. On the 6th day, however, the presence of a small number of cells of greater osmotic resistance was detected (Figure, C 6). This component increased progressively and gradually joined the main population of mature cells, until by the 12th day the curve had become similar to the original curve, showing practically a single population.

Discussion

Reticulocytes are in general more resistant to drastic osmotic haemolysis than the main population of red cells whereas the oldest red cells are most fragile (5–8). It has been demonstrated by phase microcinematography that in gradual haemolysis old cells are the first to haemolyse and young cells are the most resistant (1). The Fragiligraph which functions on the basis of gradual osmotic haemolysis (2) thus provides curves, particularly the derivative curve which represents an age population distribution of the red cells.

In the experiment described in this paper it can be seen that while the control blood maintained a normal pattern of osmotic fragility irradiation resulted in disappearance of the more resistant cells as apparent from the alteration in the fragiligram. This appears to be due to maturation of the younger cells without replacement of this age group because of erythropoietic failure, and hence there was a gradual narrowing of the age distribution in the cell population, well illustrated in the narrowing of the peak of the derivative fragiligram. In the marrow-grafted mice the evidence of production of a new population of red cells is well illustrated. Serial determinations have made it possible to detect renewed erythropoietic activity at an early stage. Because only a minute amount of blood is required for the test, the picture is not confused by the production of blood-loss anaemia.

Summary

By means of the 'Fragiligraph' it is possible to determine osmotic fragility by rapid micromethod and with high resolution as each curve is based on about 250 recordings of the degree of lysis in progressively decreasing salt concentration. The method has been applied to blood samples obtained from untreated mice, mice subjected to lethal total body irradiation and, in some of the batch, to subsequent bone-marrow transplant. The blood samples have shown changes in age population distribution as evidenced by changes in the fragiligram, especially apparent in the derivative curves. Use of the Fragiligraph provides convenient technique for demonstrating the extent of erythropoietic inhibition caused by irradiation, and for detecting erythropoietic reactivation after marrow transplant.

Zusammenfassung

Mit Hilfe des Fragiligraphen ist es möglich, die osmotische Resistenz durch eine rasche Mikromethode und mit hoher Auflösung zu bestimmen, da jede Kurve auf ca. 250 Bestimmungen des Hämolysegrades in fallenden Salzkonzentrationen beruht. Die Methode wurde angewendet auf Blutproben von unbehandelten Mäusen, von Mäusen nach letaler Ganzkörperbestrahlung und bei einigen derselben nach nachheriger Knochenmarkstransplantation. Die Blutproben zeigten Veränderungen der Altersverteilung der Population, wie sie in den Abweichungen im Fragiligramm und besonders deutlich aus den Kurvenabweichungen hervorgehen. Die Verwendung des *Fragiligraphen* ermöglicht eine Beurteilung des Ausmaßes der erythropoetischen Hemmung durch Bestrahlung und den Nachweis einer Reaktivierung der *Erythropoese* nach Knochenmarkstransplantation.

Résumé

A l'aide du fragiligraphe il a été possible de déterminer la fragilité osmotique par une microméthode rapide et d'un grand pouvoir de discrimination, chaque courbe reposant sur à peu près 250 déterminations du degré de lyse dans une concentration de sel allant diminuant. La méthode a été appliquée à des échantillons de sang prélevés

sur des souris non traitées, sur des souris ayant été soumises à une irradiation à dose léthale du corps entier et sur quelques unes de celles-ci après la transplantation consécutive de moelle osseuse. Les échantillons de sang montrèrent des changements dans la distribution de l'âge de la population, changements qui furent mis en évidence par des modifications du « fragigramme » spécialement apparentes dans les dérivées. L'emploi du « fragigraphe » rend possible une appréciation de l'importance de l'inhibition érythropoïétique causée par l'irradiation et la détection d'une réactivation de l'érythropoïèse après la transplantation de moelle osseuse.

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Separation of Viable Leukocytes from Normal Human Blood*

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To obtain normal human leukocytes for certain studies involving cell culture, our laboratory needed a method that would meet the following criteria high viability of the leukocytes, freedom from erythrocytes, and as large a yield as possible Best results were provided by a combination of several methods developed in other laboratories. To enhance the sedimentation of erythrocytes, we use polyvinylpyrrolidone (PVP) employed for this purpose first by Robineaux et al (9) in 1949 and subsequently by many investigators (13) For further purification of leukocyte suspensions, we lyse the remaining erythrocytes with streptolysin-O according to a modified version of the technique described by Malmgren et al. (5)

Materials and Methods

Blood from normal person is treated with 25 units of heparin (e.g. Sodium Heparin, Upjohn) per ml of blood. A leukocyte count is done on the sample and the rest of the blood is transferred to one or two tall, slender separatory funnels. (All glassware including syringes, funnels, and centrifuge tubes, was siliconized.) T each 10 ml aliquot of blood is added 40 ml of cold Earle buffered salt solution (EBSS) with pH of 7 4 and to this is added 50 ml of 3.5 solution of PVP (Polyvinylpyrrolidone K 30, Oxford Laboratories, approx. mol. weight 40000) in EBSS. The blood-EBSS-PVP mixture in each funnel is mixed well by pipetting it up and down few times, and the funnel is left standing upright 4 °C for 1 h.

The sedimented erythrocytes, approximately 5 of the total column of fluid, are drawn off through the bottom of the separatory funnel. The supernatant, containing leukocytes and usually somewhat contaminated with erythrocytes, is transferred to centrifuge tubes and is diluted 1 1 with cold EBSS. The cells are spun for 10 min 250 G in refrigerated centrifuge and the resulting cell pellets are combined and resuspended in 10 ml of cold EBSS T this suspension is added 140 mg of streptolysin-O (Difco) dissolved in 10 ml of EBSS. The tubes are shaken gently in water bath 37 C

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for 1 to not more than 2 min. An increase in the transparency of the cell suspension indicates that the red cells are lysed. After 20 ml of cold EBSS is added to the tube, the cells are spun down in refrigerated centrifuge t 160×G for 5 min. The leukocyte pellet is resuspended in the desired medium. W usually prefer culture medium RPMI #906 (7) supplemented with 5% fetal calf serum (FCS) A viability count, using exclusion of trypan blue, is performed.

The principal test to determine the viability of the leukocytes was measurement of their oxygen uptake (11) by means of standard Warburg manometry Varying amounts of cells, depending on the amounts available from individual samples, were suspended in 6 to 8 ml of the phosphate-buffered medium RPMI #906 supplemented with 5% FCS. After 20 min of equilibration with tmospheric air oxygen uptake was measured for 2 h. Q_{O_2} was expressed as µl of oxygen taken up per 10×10^6 cells per hour

Short-term cultures (3 to 5 days) were initiated from third of the samples of separated leukocytes. Cells were inoculated in Earle T-flasks containing culture medium RPMI #906 supplemented with 5% FCS and including 0.01 ml of phytohemagglutinin P (Difco) per ml. The cultures were observed for attachment of cells, for ameboid movements, and for change in the pH of the medium. I some instances, chromosome slides were prepared in order to demonstrate mitoses. After 72 h of incubation, 10 µg of Colcemide (Desacetylmethylcolchicine, Ciba) per ml of medium was added to the culture, and the cells were harvested 14 h later by scraping them off the glass, subjecting them to brief treatmen with hypotonic medium and fixing them with 1 3 mixture of glacial acetic acid and absolute methanol. Air-dried slides were prepared and Giemsa stained.

Results

The percentage of viable cells recovered ranged from 25 up to 84 / of the number of leukocytes initially present in the blood sample the mean recovery rate was 57 / In most instances, the viability of the cells recovered was more than 89 / on the basis of trypan blue exclusion. From examination of Giemsa-stained smear slides or from observation of the cells in the counting chambers, it was always quite obvious that the ratio of erythrocytes to leukocytes was far below 1 1 more likely 1 50 or less.

In short term cultures, attachment of cells to the glass was observed after a few hours of incubation at 37 C. Ameboid movements were obvious, as was intracellular streaming of the cytoplasm. During the next few days, a falling pH in all cultures provided evidence of active cell metabolism. In the instances where chromosome slides were prepared, numerous mitoses were observed. Oxygen uptake was measured for 9 of the 15 samples of leukocyte suspension prepared The Q_{O_2} values ranged from 1 17 to 13.3 (Table I) which is in good accord with similar measurements done by FALLON *et al* (3) and RABINOWITZ (8) on leukocyte suspensions prepared from normal human blood by other methods.

Table I
Separation of leukocytes from normal blood.

Sex of donor	Vol. of sample (ml)	wbc count (wbc/µl)	Total wbc in sample (10^6)	Recovered leucocytes: Total wbc (x 10^6)	% recovered	viable (trypan blue)	Q_{O_2}	Culture	Attachment	Ameboid movement
m	20	2790	55.8	22.2	41	96				
f	20	5550	111.0	66.9	60	92				
m	10	7520	75.2	19.5	5	99	13.3			
m	20	2070	41 4	16.4	40	95				
f	20	6610	132.2	98 8	75	94				
f	20	6390	127.8	70.6	55	94				
f	20	5750	115.0	56.1	51		2.0			
f	20	4320	68.4	37.3	54		2.0			
m	20	5800	116.0	122.0	100					
f	20	7140	142.8	105.2	74		1.2			
f	10	4480	44.8	24.7	55	84	8.4			
m	10	2710	27 1	15 9	58	84	7 4			
m	10	6620	66.2	47.2	71	89	3.3			
f	10	5370	53.7	20.0	37	91	8.7			
m	10	6050	60.5	50.0	84	89	1.9			

Discussion

Our method for separating leukocytes from normal whole blood utilized PVP for rapid sedimentation of most of the erythrocytes, followed by a short exposure to streptolysin-O for lysis of the remaining erythrocytes. The method meets our criteria since the leukocytes retain a high viability and a reasonable percentage of the cells are recovered with very little erythrocyte contamination. Streptolysin-O is known to be injurious to polymorphonuclear leukocytes when it is used according to the method described by MALMOREN *et al* (5) in a concentration of 16 mg/ml at 39 C for 10 min, it lyses large amounts of polymorphonuclear leukocytes. In a concentration of 7 mg/ml at 37 C for 1 to not more than 2 min, as in our method, streptolysin-O has caused no observed decrease in the number of polymorphonuclear leukocytes. No specific measurements were performed to determine the degree of erythrocyte contamination in the preparations, as it was obvious from microscopical observation of the leukocyte suspensions that only a very small number of erythrocytes remained after the separation procedure.

The yields obtained seem reasonable in comparison with those of other methods recorded in the literature (1–4 6, 8, 10 12 14)

A mean of 57 / of the leukocytes initially present in the samples could be recovered. This rather good percentage of recovery was aided to some extent by diluting the initial 10 ml of blood with *90 ml of fluid (essentially EBSS) before sedimentation.*

A slight tendency toward aggregation of the leukocytes could sometimes be observed, but brief and gentle pipetting of the cell suspensions would easily break up any clumps of cells. In a few instances where leukocyte pellets had been stored at 4 °C for 24 or 48 h, an appreciable amount of clumping was seen. In these instances, it was difficult to break up the clumps mechanically In such instances it may be of advantage to add small amounts of streptokinase-streptodornase, as has been done by FALLON *et al* (3)

The viability studies were based primarily on measurements of the leukocytes uptake of oxygen after separation was completed The Q_O values obtained compare favorably with those obtained by other investigators after separation of leukocytes from normal blood (2 3 8, 11) Such results do not necessarily imply that alterations in the cells do not occur during the separation procedure. It seems reasonable to conclude, however that the active respiration measured, as well as the demonstrated ability of the cells to multiply during short term culture, indicates that the separation procedure induced no gross alterations in the cells, and certainly no serious loss of viability Attachment of the cells in culture and the occurrence of ameboid movements also support the conclusion that leukocytes not only tolerate our method of separation quite well, but may be generally quite resistant to the unphysiologi conditions involved.

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Summary

A method for the separation of viable leukocytes from normal human blood is described. It is based on sedimentation of the erythrocytes with polyvinylpyrrolidone and subsequent lysis of the remaining erythrocytes with streptolysin-O A mean of 57 of the leukocytes initially present in the samples could be recovered The ratio of erythrocytes to leukocytes was far below 1 1 more likely 1 50 or less after completion of the separation. The rather good percentage of recovery was aided to some extent by diluting the initial blood sample 1 10 before the sedimentation Viability studies after the separation was completed included measurements of the leukocytes uptake of oxygen, dyeexclusion tests, observation of division in short-term cultures, as well as attachment of the cells and ameboid movements All parameters thus observed indicated that the separation procedure induced no gross alterations in the cells, and certainly no serious loss of viability

Zusammenfassung

Es wird eine Methode zur Abtrennung lebender Leukozyten aus normalem menschlichem Blut beschrieben. Sie beruht auf einer Sedimentation der Erythrozyten mit Polyvinylpyrrolidon und nachfolgender Lyse der verbleibenden roten Blutkörperchen mit Streptolysin O. Im Mittel konnten 57% der ursprünglich in den Proben vorhandenen Leukozyten gewonnen werden. Das Verhältnis von Erythrozyten zu Leukozyten lag weit unter 1 : 1, es betrug eher 1 : 50 oder weniger nach beendeter Abtrennung. Die gute Ausbeute wurde bis zu einem gewissen Grade unterstützt durch Verdünnung der ursprünglichen Blutprobe im Verhältnis 1 : 10 vor der Sedimentierung. Untersuchungen der Lebensfähigkeit nach vollzogener Abtrennung betrafen Messungen der Sauerstoffaufnahme durch die Leukozyten, Farbstoffausschlußtests, Feststellung von Teilungen in kurzfristigen Kulturen, sowie Haftung und amöboide Bewegungen der Zellen. Aus diesen Kriterien ging hervor, daß das Verfahren keine groben Zellveränderungen und sicher keine wesentliche Beeinträchtigung der Lebensfähigkeit zur Folge hat.

Résumé

Une méthode servant à la séparation de leucocytes viables du sang humain normal est décrite. Elle repose sur la sédimentation des érythrocytes en présence de polyvinylpyrrolidon et sur la lyse consécutive des érythrocytes restant au moyen de streptolysine O. En moyenne 57% des leucocytes initialement présents dans les échantillons de sang prélevé ont pu être récupérés. Le nombre relatif des érythrocytes et des leucocytes était très en dessous de 1 : 1, plutôt de 1 : 50 ou moins encore, la séparation terminée. Le pourcentage assez élevé de récupération a été en partie amélioré par la dilution de 1 : 10 avant la sédimentation des prélèvements de sang. La séparation une fois terminée, les examens de viabilité comprenaient des déterminations de l'absorption de l'oxygène par les leucocytes, des tests d'exclusions de colorants, l'observation de la division dans des cultures de courte durée, ainsi que de l'adhésion des cellules et de leurs mouvements amoeboïdes. Ces critères mirent en évidence que la méthode décrite ne provoque pas d'altérations grossières des cellules ni de diminution importante de leur viabilité.

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Electron Microscopic Observations on the Finer Structure of Basophilic Granulocytes of Newt (Triturus pyrrhogaster)

O TAKEOKA, S MATSUI AND T ASHIHARA

Recently it has been reported that cytoplasmic membranes are deformed by osmium fixation (1) We have had a similar ex perience in examining the granules of basophilic granulocyes of newt blood.

The presence or absence of these structures in the granules of the tissue mast cells and the basophilic blood leucocytes of mammals is important therefore we report our results on the blood granulocytes of newt, even though the results obtained in amphibians cannot directly be applied to the mammals.

Material and Method

One drop of newt blood was fixed in the fixation fluid in which the blood made thin layer less than 0.5 mm in thickness. I one series, the blood was fixed in Caulfield' I osmic acid fixative for one hour I another series, the blood was fixed in 5 glutaraldehyde solution buffered at pH 7 4 and added with saccarose t the room temperature for one hour and post-fixed in Caulfield' fixative for one hour The specimens were then dehydrated in ethanol, embedded in Epon 812, cut with Porter Blum microtome and examined with Hitachi HU 11 electron-microscope.

Results and Discussion

In the specimens fixed with osmic acid and stained with uranyl acetate there was a distinct structure in the granules which consisted of bundles of needle-like crystals (Fig 1) The remaining areas in the granules contained a small amount of minute particles In some granules polygonal structures were visible, suggesting a cross section of the bundle of crystals. A few of granules showed homogenously fine granular content. Corresponding to these inner structures found electron microscopically rod-like metachromatic ma

terial was seen in the granules on the section cut from the same epon block and stained with toluidine blue (Fig 2) Except for the rod like metachromatic structure, the content of the granules remained unstained. When the sections for the electron microscopic examination were stained with lead hydroxyde, the needle-like crystals were not stained, being seen as empty narrow spaces, and the remaining parts of the granules appeared more dense with fine granular material Moreover these fine granular material seemed to be reduced in density proportionally to the distance from the Golgi area. The granules nearest to the Golgi area had no crystals.

In the specimens fixed with glutaraldehyde followed by post fixation with osmium, we could not find any crystal structure in the granules. The content of granules was either homogenous and amorphous (Gh. in Fig 3) or fine reticular (Gr in Fig. 3) and filled each granule entirely In some granules both elements were present together Corresponding to these inner structures seen electron microscopically a section from the same epon block, when stained with toluidine blue showed a homogenous metachromatic material filling the granules.

It should be mentioned here that with routine staining methods on smear specimens or when observed in a native preparation with a phase contrast microscope the content of the granules appeared as a homogenous material without any significant inner structures. *Therefore the pattern of staining of the granules from* the electron-microscopic material fixed with glutaraldehyde was almost identical with those of the routine smear specimens corresponding more with the granules of the living granulocytes. The conclusion was warranted that the crystal structure in the granules of the specimens fixed with osmium tetraoxide is an artefact owing to the osmium fixation.

According to Smith (2) the preservation of the cytoplasmic organells of tissue mast cells of rat or mouse was very difficult in the electron-microscopic material We had similar experiences on the mast cells of rat or mouse, and also on the granulocytes of newt blood, when osmic acid was used as a fixati e. However in the *glutaraldehyde-fixed material of newt blood, the preservation of* the Golgi vesicles, endoplasmic reticulum and mitochondria was far better than in those of osmium fixed material

In the light microscopic studies, many authors have described differences between basophilic leucocytes and tissue mast cells of mammals. Although in general differences in the cell size and the numbers of granules between basophilic leucocytes and the tissue mast cell do exist, these differences are quantitative and not qualitative they do not appear fundamental. MAXIMOW (3) who described the differences between both cell types, stated that in some instances it was difficult to differentiate them. On the other hand one of us (4) observed the mast cells with a scanty cytoplasm and very few granules in the lymph nodes of rat both in normal and pathological states This feature has been described as specific to the blood basophilic leucocytes. In the pathological state, where a marked increase of mast cells was observed in the mediastinal lymph nodes, distinct mast cells appeared in the peripheral blood. As to the form of the nucleus, one of us (5) already reported that the nucleus of mast cells in the peritoneal fluid of mouse can show a simple or complicated indentation which has been described also as specific to the blood basophilic leucocytes. Recently COWDEN *et al* (6) have reported the differences between both types of basophilic cells in newt Here again the differences seem to be quantitative rather than qualitative

From the beginning of the electron microscopic examinations on the mast cells and basophilic leucocytes, the existence or absence of inner structure within the granules of both types of basophilic granulocytes has been an interesting and important problem.

POLICARD *et al* (7) who described the presence of an inner structure in the granules of rat tissue mast cells, believed that this is a fundamental difference which allows to differentiate tissue mast cells from basophilic leucocytes. The latter has, according to him, no such inner structure On the other hand, PEASE (8) and WINQVIST (9) described the presence of lamellar structure in the granules of rat basophilic myelocytes. SETOGUTI *et al* (10 11) described the presence of lamellar structure in the granules of newt tissue mast cells using the osmic acid fixative they believed that

Fig 3. Basophilic granulocyte of newt blood, glutaraldehyde-fixed. Note the homogenous and amorphous or fine reticular contents of the granules (40 000)

Fig 4 Basophilic granulocyte of the section from the same epon block as Fig 3, stained with toluidine blue. Note the homogenous metachromatic staining of the contents of granules (2000)



Fig. 1 Basophilic granulocyte of newt blood, osmium-fixed. Note the crystal structures in the granules (20 000

Fig. 2 Basophilic granulocyte of the section from the same epon block as Fig. 1 stained with toluidine blue. Note the rod-like metachromatic structures in the granules (×2000)

this is an essential structure in these cells. Many authors reported that there are inner structures in the granules of tissue mast cell STOECKENIUS (12) in man, HATAI *et al.* (13) in clemmys japonica, HIBBS *et al.* (14) in man (round cell) Others found an almost homogenous (granular or fine reticular) structure of the granules of tissue mast cell SMITH on hamster and rat, HATAI *et al.* on Geoclemmys reevesii and HIBBS *et al.* on man (long cell) There can be, therefore, not definitely concluded whether or not the granules of the basophilic granulocytes have an inner structure.

According to our observation the crystal structure in the granules of basophilic granulocytes of newt blood is an artefact due to osmic acid fixation Moreover the facts that there are differences in the density of content of granules in one and the same cell, which is fixed osmic acid and stained with lead hydroxide, and that the granules near to the Golgi area are free of crystal structures, suggest different stages of the development of granules.

Of course, these observations on the newt blood granulocytes can not be applied directly to those of the mammals nevertheless they indicate that the presence or absence of some crystal or lamellar structure in the granules of tissue mast cells or blood basophilic leucocytes of mammals cannot be used as criterion to differentiate both types of cells.

Summary

The basophilic granulocytes of newt blood were examined electron-microscopically with two kinds of fixation method. With osmic acid fixation there was crystal structure in the granules of the cell, but none with glutaraldehyde fixation. The granules had homogenous or fine reticular content. In comparing the toluidine blue stained epon sections with the routine smear specimens and the living granulocytes, the conclusion appears purified that the crystal structure in the granules of the basophilic granulocytes of newt blood, observed in the electron-microscopic material is an artefact owing to the osmium fixation.

Zusammenfassung

Die basophilen Granulozyten aus dem Blut des Wassermolches wurden elektronenmikroskopisch mit zwei Fixationsverfahren untersucht. Eine kristalline Struktur der Granula wurde mit Osmiumsäurefixation gefunden, jedoch nicht bei Fixation mit Glutaraldehyd. Die Granula wiesen einen homogenen oder fein-retikulären Inhalt auf. Beim Vergleich der mit Toluidinblau gefärbten Epon-Schnitte und der Routine-Ausstriche oder der lebenden Granulozyten ergab sich, daß die Kristallstruktur der basophilen Granulozyten des Wassermolches, wie sie im Elektronenmikroskop beobachtet wird, ein durch die Osmiumfixation bedingtes Kunstprodukt darstellt.

Résumé

Les granulocytes basophiles du sang de la salamandre aquatique ont été étudiés au microscope électronique à l'aide de deux modes de fixation. Une structure cristalline se trouve dans les granulations des cellules fixées à l'aide d'acide osmique, mais pas dans celles fixées à l'aide de glutaraldéhyde. Les granulations montrèrent un contenu homogène ou une fine structure réticulaire. En comparant les coupes (epon) colorées au bleu de toluidine aux frottis de routine ou aux granulocytes vivants, nous avons tiré la conclusion que la structure cristalline des granulations des granulocytes basophiles de la salamandre aquatique, comme elle apparaît au microscope électronique, est un artefact dû à la fixation à l'osmium.

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Index rerum ad Vol. 31–35

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